REMARKS/ARGUMENTS

Claims 7-11 and 13-16 are pending; Claims 1-6 and 12 are canceled. Claim 16 is withdrawn from consideration. Reconsideration of the rejections is requested.

The presently pending claims have been rejected as anticipated or made obvious by Rodan *et al.*, U.S. Patent no. 5,780,291. Applicants respectfully submit that the cited art fails to teach a method of isolating a biologically active, substantially homogeneous composition of Wnt protein comprising a lipid moiety.

Applicants respectfully submit that the cited art does not teach the presently claimed methods. It is noted that the cited document is silent as to the hydrophobic nature of Wnt proteins, and to the presence of a lipid moiety.

The Examiner has questioned the previously submitted Declaration made by Dr. Willert. Applicants wish to note that there is no reason to doubt the veracity of the statements provided in the 132 Declaration, and respectfully submit that such should be accepted in the absence of evidence to the contrary.

In particular, a question was raised as to the presumed validity of the Rodan *et al.* claims. As Applicants have previously argued, the ability of Rodan *et al.* to isolate a particular protein is not being questioned; it is the biological activity of the isolate that is lacking in the prior art description.

Certainly one cannot assume that a polypeptide must have a biological activity in order to be isolated and claimed in a patent. Indeed, the case law argues that such activity is not a required element. One may look to Appeal No. 2004-2314, Ex Parte Friedberg *et al,* which found that the ability of a peptide to act as an immunogen was sufficient utility for a patent claim. It is therefore improper to assume that the claims of Rodan *et al.* inherently claim a biologically active protein composition.

The difficulty in isolation of Wnt proteins is well-known in the art. For example, one may look to the attached review by Jeffrey Miller (Genome Biology 2001 3(1):reviews3001.1-3001.15), which states at page 2 "very little is known about the structure of Wnt proteins as they are notoriously insoluble". The attached article by Logan and Nusse (Annu. Rev. Cell Dev. Biol. 2004, 20:781-810) further discusses the point, stating at page 783 that "although Wnt proteins are secreted, difficulties in solubilizing active Wnt molecules had hindered attempts to purify the Wnts and precluded a thorough biochemical characterization of this growth factor family. The insoluble nature of Wnts has now been explained by the discovery that these proteins are

palmitoylated and are more hydrophobic that initially predicted from the primary amino acid sequence."

Applicants note the publication by the inventors of the present application in the international peer-reviewed journal, Nature. As stated in the abstract of Willert *et al.* (2003) Nature 423:448-452, "Wnt proteins are potentially important reagents in expanding specific cell types, but in contrast to other developmental signaling molceuls such as hedgehog proteins and the bone morphogenetic proteins, Wnt proteins have never been isolated in an active form. Although Wnt proteins are secreted from cells, secretion is usually inefficient and previous attempts to characterize Wnt proteins have been hampered by their high degree of insolubility."

Applicants respectfully submit that one of skill in the art is aware of these publications in well-respected journals, and would be aware that Wnt proteins are insoluble when dialyzed into PBS, and therefore the teachings of Rodan *et al.* would not lead to a composition of isolated and biologically active Wnt.

With respect to the specific Wnt protein described by Rodan *et al*, Applicants have attempted to search the scientific literature for evidence of this protein and its possible biological activity. However, a search of Pubmed and of human genome sequences has failed to yield any indication of a "wnt-x" gene; nor has Rodan *et al.* published any articles relating to wnt proteins. A BLAST sequence comparison of the protein claimed in US 5,780,291, performed against all known sequences in Genbank reveals a single match in the patents database, corresponding to the '291 patent sequence listing, however no perfect match was found in any other sequence, *e.g.* in the human genome sequence, or in the genecard database, which provides information for known human genetic loci. It seems that the Wnt-x protein may be related to Wnt-2b, with which it has 96% sequence identity.

On the basis of such a high degree of sequence similarity, Applicants submit that one of skill in the art would believe that the proposed "wnt-x" protein would share the properties of the known protein, Wnt2b, and as such would be insoluble when dialyzed against PBS in the absence of detergent. It is respectfully submitted that the cited art does not teach the isolation of biologically active Wnt protein, and does not anticipate or make obvious the claims of the present application.

Applicant respectfully requests that a timely Notice of Allowance be issued in this case.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number STAN-299.

Respectfully submitted,
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EXHIBIT 1

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THE WNT SIGNALING PATHWAY IN DEVELOPMENT AND DISEASE

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Key Words embryogenesis, cancer, β-catenin, Frizzled, stem cells

■ **Abstract** Tight control of cell-cell communication is essential for the generation of a normally patterned embryo. A critical mediator of key cell-cell signaling events during embryogenesis is the highly conserved Wnt family of secreted proteins. Recent biochemical and genetic analyses have greatly enriched our understanding of how Wnts signal, and the list of canonical Wnt signaling components has exploded. The data reveal that multiple extracellular, cytoplasmic, and nuclear regulators intricately modulate Wnt signaling levels. In addition, receptor-ligand specificity and feedback loops help to determine Wnt signaling outputs. Wnts are required for adult tissue maintenance, and perturbations in Wnt signaling promote both human degenerative diseases and cancer. The next few years are likely to see novel therapeutic reagents aimed at controlling Wnt signaling in order to alleviate these conditions.

CONTENTS

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INTRODUCTION

The field of developmental biology is rich with a history of observations predicting the existence of signaling molecules that control key events in embryogenesis (Gilbert 1991). Over the past 20 to 30 years, several families of signaling molecules such as the bone morphogenetic proteins (BMPs), the Hedgehogs, the fibroblast growth factors (FGFs), and the Wnts have been identified, and their signaling mechanisms have been elucidated. These signaling pathways are also often involved in disease, in particular cancer, reinforcing the concept that cancer is a form of development gone awry.

The Wnt family of signaling proteins participates in multiple developmental events during embryogenesis and has also been implicated in adult tissue homeostasis. Wnt signals are pleiotropic, with effects that include mitogenic stimulation, cell fate specification, and differentiation. This review summarizes our current understanding of Wnt signaling in the context of the many developmental roles of this pathway. As the volume of Wnt literature is increasing rapidly, a few aspects of current interest have been selected here, mainly focused on Wnt signaling through its receptors (Frizzleds) to β-catenin, which is often called the canonical pathway. Much work has been done recently on noncanonical pathways, which do not involve β-catenin or Wnt ligands. That topic is not covered here, but we direct readers to some recent reviews (Strutt 2003, Veeman et al. 2003). A continuing update on Wnt signaling, including figures and gene tables, can be found on the Wnt homepage http://www.stanford.edu/~rnusse/wntwindow.html.

WNT SIGNALING: AN OVERVIEW

A simple outline of the current model of Wnt signal transduction is presented in Figure 1. Wnt proteins releasedfrom or presented on the surface of signaling cells act on target cells by binding to the Frizzled (Fz)/low density lipoprotein (LDL) receptor-related protein (LRP) complex at the cell surface. These receptors transduce a signal to several intracellular proteins that include Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3), Axin, Adenomatous Polyposis Coli (APC), and the transcriptional regulator, β-catenin (Figure 1). Cytoplasmic β-catenin levels are normally kept low through continuous proteasome-mediated degradation, which is controlled by a complex containing GSK-3/APC/Axin. When cells receive Wnt signals, the degradation pathway is inhibited, and consequently β-catenin accumulates in the cytoplasm and nucleus. Nuclear β-catenin interacts with transcription factors such as lymphoid enhancer-binding factor 1/Tcell-specific transcription factor (LEF/TCF) to affect transcription. A large number of Wnt targets have been identified that include members of the Wnt signal transduction pathway itself, which provide feedback control during Wnt signaling.

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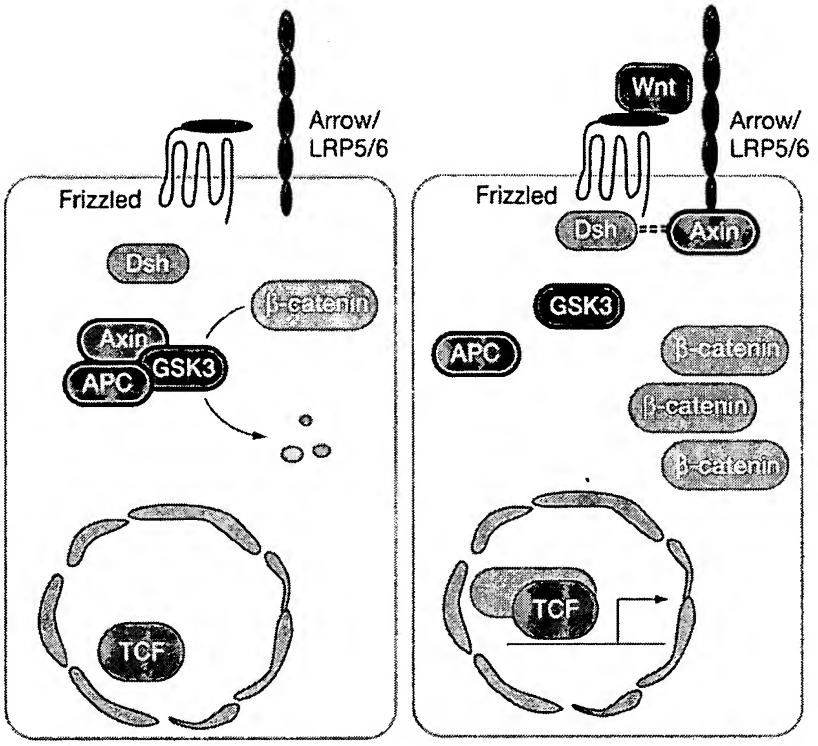

Figure 1 The canonical Wnt signaling pathway. In cells not exposed to a Wnt signal (*left panel*), β-catenin is degraded through interactions with Axin, APC, and the protein kinase GSK-3. Wnt proteins (*right panel*) bind to the Frizzled/LRP receptor complex at the cell surface. These receptors transduce a signal to Dishevelled (Dsh) and to Axin, which may directly interact (*dashed lines*). As a consequence, the degradation of β-catenin is inhibited, and this protein accumulates in the cytoplasm and nucleus. β-catenin then interacts with TCF to control transcription. Negative regulators are outlined in black. Positively acting components are outlined in color.

WNT PROTEINS ARE LIPID MODIFIED

Wnt proteins are defined by sequence rather than by functional properties. They contain a signal sequence followed by a highly conserved distribution of cysteines. Although Wnt proteins are secreted, difficulties in solubilizing active Wnt molecules had hindered attempts to purify the Wnts and precluded a thorough biochemical characterization of this growth factor family. The insoluble nature of Wnts has now been explained by the discovery that these proteins are palmitoylated and are

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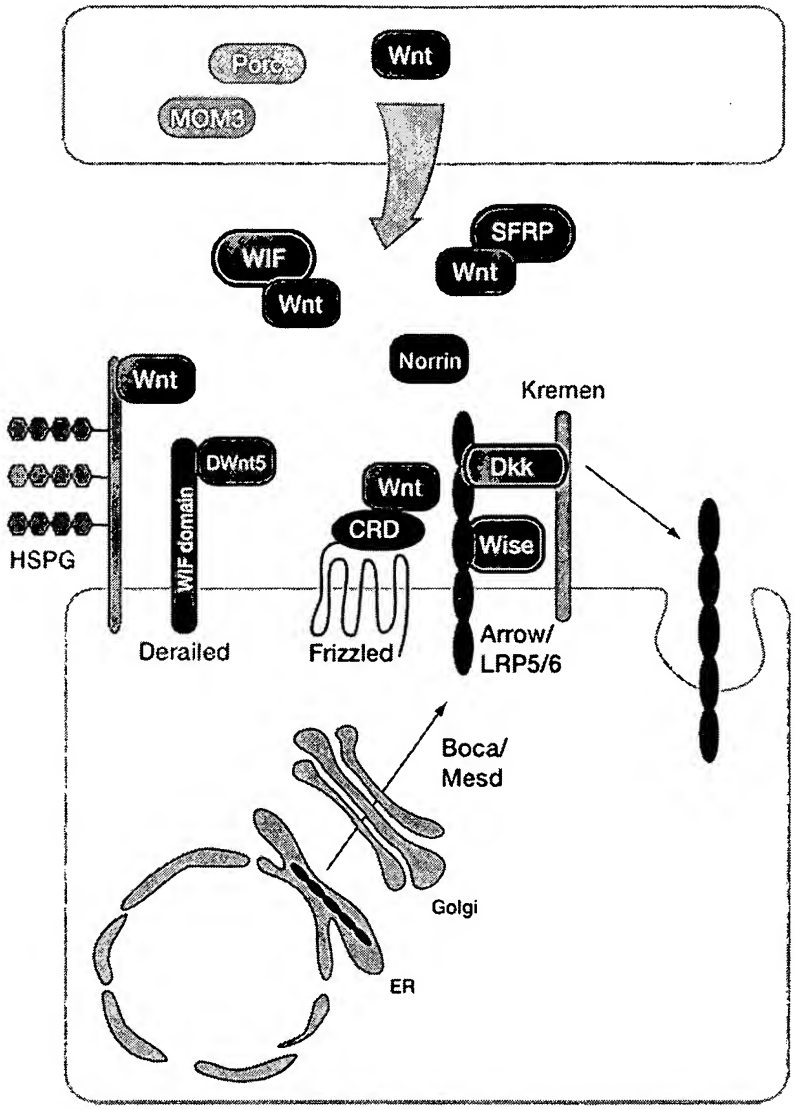
more hydrophobic than initially predicted from the primary amino acid sequence (Willert et al. 2003). The palmitoylation is found on a conserved cysteine, suggesting that all Wnts carry this modification. Mutant analysis has demonstrated that this cysteine is essential for function, and treating Wnt with the enzyme acyl protein thioesterase results in a form that is no longer hydrophobic or active, providing further evidence that the palmitate is critical for signaling (Willert et al. 2003).

The enzymes that add the palmitate to Wnts are likely to be encoded by the *porcupine* (*por*) gene in *Drosophila* (Kadowaki et al. 1996), called *mom-1* in *Caenorhabditis elegans* (Rocheleau et al. 1997). Phenotypic similarities between *wnt* and *por/mom-1* suggest that Porcupine and MOM-1 are enzymes dedicated to Wnt signaling (Kadowaki et al. 1996). These genes are required in Wnt-producing cells rather than in cells receiving the Wnt signal (Figure 2). Hofmann (2000) noticed sequence similarity between Porcupine and membrane-bound acyltransferases, enzymes that are present in the endoplasmic reticulum (ER) membrane and acylate a variety of substrates. Therefore, it is possible that *por* encodes an enzyme that catalyzes the transfer of palmitate onto Wnt. Consistent with this idea, Wingless, like Wnt3a, is also hydrophobic (Zhai et al. 2004). Wingless can associate with membranes, but both its hydrophobicity and membrane localization are lost when *O*-acyltransferase activity is inhibited biochemically or when Porcupine is eliminated genetically. These data support the hypothesis that Porcupine is a key regulator of both Wnt lipidation and membrane targeting. Interestingly, Hedgehog proteins also carry an N-terminal palmitate that is essential for signaling. The addition of this palmitate is thought to be catalyzed by Skinny-hedgehog which, like Porcupine, resembles acyl-tranferases (Chamoun et al. 2001).

Figure 2 Wnt pathway molecules that facilitate secretion or presentation of Wnt proteins or that modulate Wnt signaling levels. Porcupine (Por) is an ER protein required in Wnt-producing cells, and it may attach a palmitate to Wnt. In *C. elegans*, the MOM-3 gene product (not yet identified molecularly) may assist in the production or release of active Wnt. In vertebrates, Wnt proteins are inhibited by direct binding to either secreted frizzled-related protein (SFRP) or Wnt inhibitory factor (WIF). SFRP is similar in sequence to the cysteine-rich domain (CRD) of Frizzled, one of the Wnt receptors. The Wnt inhibitors Dickkopf (Dkk) and Wise bind to the Wnt coreceptors Arrow and LRP. Dkk also interacts with Kremen to down-regulate LRP/Arrow from the cell surface. In *Drosophila*, Wnt can bind to the tyrosine kinase receptor Derailed [related to tyrosine kinases (RYK) in mammals]. This receptor has a domain similar to WIF. Heparin-sulfated forms of proteoglycans (HSPG) are also involved in Wnt reception or transport. Boca/Mesd is specifically required for the transport of Arrow/LRP in the ER. A novel Frizzled ligand, Norrin, has also been identified. Similar to Wnt, Norrin bound to LRP and Frizzled can stimulate the canonical signaling pathway. Negative regulators are outlined in black. Positively acting components are outlined in color.



Although palmitoylation is integral to Wnt signaling, its precise function is not known. Overexpression of Wingless in *Drosophila* can partially circumvent the need for *por* function (Noordermeer et al. 1995) and, similarly, Wnt mutant gene constructs lacking the palmitoylation site can produce an attenuated signal when overexpressed in cells (Willert et al. 2003). One explanation for these observations

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is that the lipid moiety targets Wnts to membranes but its absence can be overcome by high protein concentrations.

TRANSPORT OF WNT PROTEINS BETWEEN CELLS

The detection of Wnt proteins in many tissues has been problematic owing to lack of suitable antibody reagents, but antibody staining of Wingless has demonstrated significant spread of the protein in *Drosophila* imaginal discs (Cadigan et al. 1998, Strigini & Cohen 2000). These data have indicated that the Wnts, such as Wingless in *Drosophila*, function as concentration-dependent long-range morphogenetic signals that can act on distant neighbors (Cadigan et al. 1998, Strigini & Cohen 2000, Zecca et al. 1996). This raises the questions of whether palmitoylated Wnt molecules are actively transported, how Wnts are released from cells, and how Wnts move over long distances. Are Wnts always tethered to membranes, even when shuttled between cells? Alternatively, are there carrier molecules that bind to the palmitate? Vesicle-based transport outside of cells has been proposed to exist in *Drosophila* wing imaginal discs. The vesicles, termed argosomes, might carry Wg protein as cargo (Greco et al. 2001). Wnts may also be transported by cytonemes, long thin filopodial processes that might carry Wnts and other growth factors away from signaling cells (Ramirez-Weber & Kornberg 1999). There is no evidence for specific exporters of Wnt molecules, although *mom-3*, identified in *C. elegans*, is required in Wnt-producing cells (Figure 2) (Rocheleau et al. 1997). This gene (also called *mig-14*) remains to be characterized molecularly.

Once Wnt proteins are secreted, a number of binding partners can modulate their activity. Emerging evidence suggests a role for HSPGs in the transport or stabilization of Wnt (Figure 2). In *Drosophila*, absence of *Dally*, an HSPG (Lin & Perrimon 1999, Tsuda et al. 1999), and mutations in genes encoding enzymes that modify HSPG (Baeg et al. 2001, Lin & Perrimon 2000) generate phenotypes similar to *wingless* mutants. HSPGs have been postulated to function as coreceptors on target cells (Lin & Perrimon 1999), but cultured cells lacking Dally can respond to Wg (Lum et al. 2003). HSPGs may therefore stabilize Wnt proteins or aid in its presentation or movement between cells.

Secreted Wnts may also bind members of the SFRP family. These are secreted proteins that resemble the ligand-binding domain of the Frizzled family of Wnt receptors (Hoang et al. 1996, Rattner et al. 1997). Alternatively, Wnts may bind WIF proteins, which are secreted molecules resembling the extracellular portion of the Derailed/RYK class of transmembrane Wnt receptors (Hsieh et al. 1999a) (Figure 2). In general, both SFRPs and WIFs are thought to function as extracellular Wnt inhibitors (Bafico et al. 1999, Dennis et al. 1999, Hsieh et al. 1999a, Leyns et al. 1997, Salic et al. 1997, Uren et al. 2000, Wang et al. 1997). However, it has not been ruled out that these proteins, depending on expression levels or cellular context, promote Wnt signaling by protecting Wnts from degradation or by facilitating Wnt secretion or transport (Uren et al. 2000).

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WNT RECEPTORS AND THEIR INTERACTIONS WITH EXTRACELLULAR INHIBITORS

Genetic and biochemical data have demonstrated that the Fz proteins are the primary receptors for the Wnts (Bhanot et al. 1996) (Figure 2). Fzs are seven-transmembrane receptors with a long N-terminal extension called a cysteine-rich domain (CRD). Wnt proteins bind directly to the Fz CRD (Bhanot et al. 1996, Dann et al. 2001, Hsieh et al. 1999b). In *Drosophila* and in cell culture, overexpression of the DFz2 receptor fails to activate Wnt signaling unless its cognate ligand, Wingless, is present (Bhanot et al. 1996, Rulifson et al. 2000), suggesting that Fz activation during canonical signaling is ligand dependent.

In addition to Wnt/Fz interactions, Wnt signaling also requires the presence of a single-pass transmembrane molecule of the LRP family (Figure 2), identified as the gene *arrow* in *Drosophila* (Wehrli et al. 2000) and as *LRP5* or *6* in vertebrates (Pinson et al. 2000, Tamai et al. 2000). The transport of LRP from the ER to the cell surface requires a specific accessory molecule called Boca in *Drosophila* and Mesd in mice (Culi & Mann 2003, Hsieh et al. 2003); mutations in these genes produce phenotypes similar to loss of Arrow/LRP itself. It has been proposed (Tamai et al. 2000) that Wnt molecules bind to LRP and Frizzled to form a receptor trimeric complex, although this has not been observed for Wingless and Arrow in *Drosophila* (Wu & Nusse 2002). Nevertheless, the importance of LRP is underscored by the finding that potent, extracellular inhibitors of Wnt signaling such as Wise (Itasaki et al. 2003) and Dickkopf (Glinka et al. 1998) bind to LRP. The best characterized of the secreted Wnt-signaling inhibitors are the Dickkopf (Dkk) proteins. Dkks have not been found in invertebrates, but mice and humans have multiple Dkk genes (Krupnik et al. 1999, Monaghan et al. 1999). Dkk1, in particular, is a potent Wnt-signaling inhibitor (Glinka et al. 1998). It binds to LRP with high affinity (Bafico et al. 2001, Mao et al. 2001a, Semenov et al. 2001) and to another class of transmembrane molecules, the Kremens (Mao & Niehrs 2003, Mao et al. 2002). By forming a complex with LRP and Kremen, Dkks promote the internalization of LRP, making it unavailable for Wnt reception. The inhibitory function of Dkks depends on the presence of appropriate Kremen proteins. Dkk2 requires Kremen2 in order to inhibit Wnt signaling and cannot function with Kremen1 to down-regulate the Wnt signal (Mao & Niehrs 2003). Likewise, Kremen2 promotes the inhibitory activity of Dkk4 (Mao & Niehrs 2003).

In addition to binding the Wnts, Frizzled can also interact with another ligand. Norrin, a protein with no discernable sequence similarity to the Wnts, binds with high affinity to the Frizzled-4 CRD (Xu et al. 2004). Together with LRP, Frizzled-4 and Norrin can activate the canonical signaling pathway. The ability of Wnt receptors to interact with multiple ligands underscores the pleiotropy of Frizzled activity, and it is possible that there are yet additional ligands for this receptor family.

Recent data show that Derailed, another Wnt receptor, is entirely distinct from the Frizzleds (Figure 2). The Derailed receptor is a transmembrane tyrosine

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kinase belonging to the RYK subfamily. Dwnt-5 is a regulator of axon guidance in the *Drosophila* central nervous system (CNS), and embryos mutant for Dwnt-5 resemble those lacking Derailed, i.e., they display misrouting of neuronal projections across the midline (Yoshikawa et al. 2003). The Derailed extracellular region contains a Wnt-interacting WIF domain (Hsieh et al. 1999a) that can bind to the DWnt-5 protein (Yoshikawa et al. 2003), indicating that Derailed is a DWnt-5 receptor in the CNS. How Derailed signals are transduced is not clear; the Derailed kinase domain appears dispensable for function (Yoshikawa et al. 2001), and the possibility that signaling involves a coreceptor has not been excluded. In vertebrates, both Wnt4 and Wnt5 have been implicated in axon guidance (Hall et al. 2000, Lyuksyutova et al. 2003). Wnt4 appears to signal in this context through a Frizzled. Whether a RYK binds to Wnt5 is not known because the Wnt5a receptor has not been identified. It will be interesting to determine whether Fz/LRP and RYK receptors function in the same tissues and cellular processes and, if so, whether Wnts simultaneously contact Fz/LRP and RYK-like kinases or stimulate RYK and Fz/LRP receptors in parallel pathways.

HOW DO THE WNT RECEPTORS SIGNAL?

The observation that Fzs contain seven transmembrane regions has led to the suggestion that Wnt binding might reconfigure the Fz transmembrane domains, as occurs in other heptahelical receptors. How a reconfigured Fz receptor couples to downstream effectors is not understood. Dsh, a ubiquitously expressed cytoplasmic protein (Lee et al. 1999, Yanagawa et al. 1995), functions cell autonomously and genetically upstream of proteins such as β-catenin and GSK-3 (Noordermeer et al. 1994). It has been postulated that Dsh transduces the Wnt signal into the cell through a direct binding between Dsh and Fz (Figures 2 and 3). Consistent with this idea, Dsh can interact with Fz directly (Chen et al. 2003, Wong et al. 2003) through a C-terminal cytoplasmic Lys-Thr-X-X-X-Trp motif in Fz that is required for Fz signaling (Umbhauer et al. 2000). Wnt signaling also leads to differential phosphorylation of Dsh (Yanagawa et al. 1995), and this process is mediated by several protein kinases, of which Par1 is the most likely Wnt-regulated candidate (Sun et al. 2001). Some questions that remain are whether Wnt binding to Fz regulates a direct Fz-Dsh interaction, how Dsh phosphorylation is controlled, and how phosphorylated Dsh functions in Wnt signal transduction.

Similar to Fz, LRP may also interact with a cytoplasmic component of the Wnt-signaling pathway. The cytoplasmic tail of LRP contains several Pro-Pro-Pro-(SerTrp)Pro [PPP(S/T)P] motifs that can become phosphorylated following Wnt stimulation (Tamai et al. 2004). Because LRP can interact with the cytosolic protein Axin (Mao et al. 2001b, Tolwinski et al. 2003) (Figures 2 and 3), Wnts are thought to induce the phosphorylation of LRP on a PPP(S/T)P motif, thus allowing the docking of Axin to the LRP cytoplasmic tail.

Both Dsh and Axin contain a stretch of amino acids called the DIX domain. DIX domains of Axin can homodimerize (Hedgepeth et al. 1999, Hsu et al. 1999, Sakanaka & Williams 1999), and Dsh, and a *Xenopus* Axin, XARP, can heterodimerize through their DIX domains (Itoh et al. 2000). It is possible, therefore, that Wnt binding of Fz and LRP promotes direct interaction between Axin and Dsh through their DIX domains, reconfiguring the protein complex that regulates β-catenin levels in the cell (Figure 3).

Because most seven-transmembrane receptors signal through heterotrimeric G proteins, it is reasonable to ask whether Fzs interact with G proteins as well.

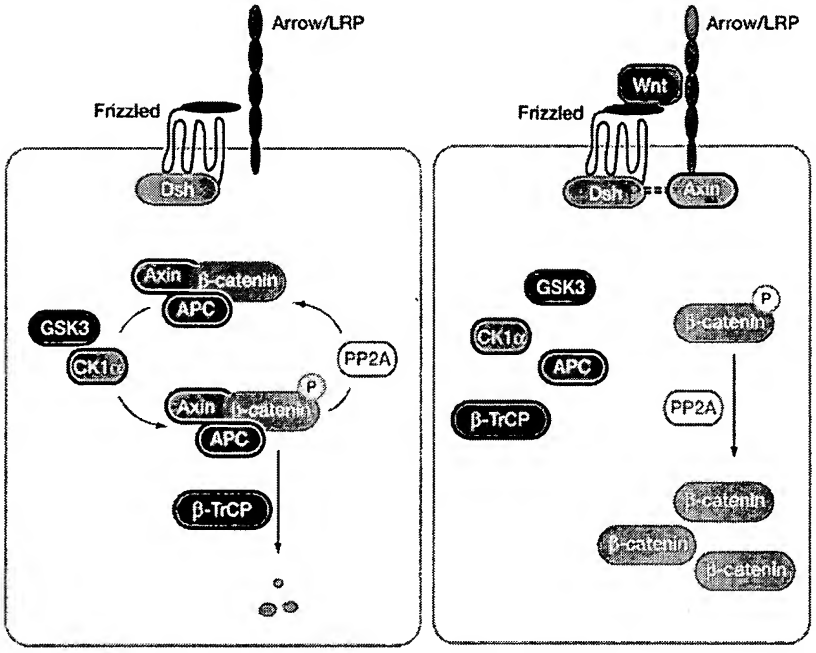

Figure 3 Cytoplasmic components of the Wnt signaling pathway, In naïve cells (*left panel*), β-catenin forms a complex with Axin and APC. Axin acts as a scaffold for the protein kinases CK1a and GSK-3, and the PP2A protein phosphatase. PP2A may act on Axin as well as other on substrates in the Axin/APC complex. After phosphorylation by GSK-3 and CK1a, β-catenin is degraded by ubiquitination involving interactions with Slimb/β-TrCP. After binding of the Wnt ligand (*right panel*), the Fz and LRP receptors recruit Dsh and Axin to the membrane where they may interact with each other (*dashed lines*). Wnt signaling leads to inhibition of β-catenin degradation and its accumulation. As a result, β-catenin is stabilized in the cytoplasm and is no longer degraded. Negative regulators are outlined in black. Positively acting components are outlined in color.

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The most direct test of this hypothesis would be to add a known Wnt ligand to cells expressing the cognate Frizzled receptor and to examine the immediate consequences. Because Wnt proteins have been difficult to isolate, experiments along these lines have utilized chimeric Fz receptors that can be activated by a non-Wnt ligand. Chimeric receptors consisting of the intracellular loops of rat Fz1 or rat Fz2 and the transmembrane and exofacial regions of β-adrenergic receptor could be activated by a β-adrenergic agonist and appear to signal through G proteins of the Go, Gq, and Gt classes (X. Liu et al. 1999, Liu et al. 2001). Whether natural Fz molecules can couple directly to heterotrimeric G proteins, however, remains to be tested.

WNT SIGNALING WITHIN THE CYTOPLASM

A hallmark of Wnt pathway activation is the elevation of cytoplasmic β-catenin protein levels. In the absence of Wnt signaling, β-catenin is phosphorylated by the serine/threonine kinases, casein kinase Iα(CKIα) (Amit et al. 2002, Liu et al. 2002, Yanagawa et al. 2002) and GSK-3 (Yost et al. 1996). The interaction between these kinases and β-catenin is facilitated by the scaffolding proteins, Axin and APC (Hart et al. 1998, Kishida et al. 1998). Together, these proteins form a β-catenin degradation complex that allows phosphorylated β-catenin to be recognized by β-TrCP, targeted for ubiquitination, and degraded by the proteosome (Aberle et al. 1997, Latres et al. 1999, C. Liu et al. 1999) (Figure 3).

Activation of Wnt signaling inhibits β-catenin phosphorylation and hence its degradation. The elevation of β-catenin levels leads to its nuclear accumulation (Tolwinski & Wieschaus 2004, Miller & Moon 1997, Cox et al. 1999) and complex formation with LEF/TCF transcription factors (van de Wetering et al. 1997, Behrens et al. 1996, Molenaar et al. 1996). β-catenin mutant forms that lack the phosphorylation sites required for its degradation are Wnt unresponsive and can activate Wnt target genes constitutively (Munemitsu et al. 1996, Yost et al. 1996). β-catenin, APC, and Axin mutations that promote β-catenin stabilization are found in many different cancers, indicating that constitutive Wnt signaling is a common feature in many neoplasms (reviewed in Giles et al. 2003).

Wnt signals might influence the cytoplasmic proteins that regulate β-catenin stability through several mechanisms. Reception of a Wnt signal could trigger the recruitment of Axin either to LRP or to Frizzled-bound Dsh, removing Axin from the destruction complex to promote β-catenin stabilization (Cliffe et al. 2003, Tamai et al. 2004). Protein phosphatases also regulate β-catenin stability. PP2A, for example, is required for the Wnt-dependent elevation of β-catenin levels (J. Yang et al. 2003) and can bind Axin (Hsu et al. 1999), suggesting that it might function to dephosphorylate GSK-3 substrates. How PP2A activity is regulated by Wnt signals is not known. Finally, Dsh can interact with the destruction complex through the GSK-3 binding protein, GBP/Frat (Jonkers et al. 1997, Salic et al. 2000, Yost et al. 1998). Frat may promote the dissociation of GSK-3 from

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the degradation complex and prevent the phosphorylation of β-catenin (Li et al. 1999), but how Wnt signals regulate this event is not clear. Current approaches to elucidate the mechanisms of β-catenin regulation include attempts to determine the structure of the degradation complex. There are now crystallographic structures of Axin contacting β-catenin (Xing et al. 2003) and Axin bound to APC (Spink et al. 2000). However, the composition of the destruction complex and the stoichiometry of the various components have not been fully resolved. Recent data suggest that the number of Axin molecules in cells is much lower (5000-fold) than other proteins in the complex (Lee et al. 2003). Axin, therefore, may be a limiting component of the Wnt signaling cascade that, as a key scaffolding molecule, may promote the rapid assembly and disassembly of Wnt pathway components to regulate β-catenin stability in the cell. Given that Dsh, APC, GSK-3, and β-catenin participate in other signaling events, low Axin levels may also help to insulate the Wnt pathway from changes in the abundance of the other Wnt signaling components when they participate in different signaling processes. Further detailed stoichiometric analyses will provide deeper insights into the exact nature of the β-catenin destruction complex and the mechanisms that regulate its function.

SIGNALING IN THE NUCLEUS

The increased stability of β-catenin following Wnt signaling leads to the transcriptional activation of target genes mediated by β-catenin interactions with the TCF/LEF DNA-binding proteins (Figure 4) (van de Wetering et al. 1997, Behrens et al. 1996, Molenaar et al. 1996). In the absence of the Wnt signal, TCF acts as a repressor of Wnt/Wg target genes (Brannon et al. 1997) by forming a complex with Groucho (Cavallo et al. 1998). The repressing effect of Groucho is mediated by interactions with histone deacetylases (HDAC), which are thought to make DNA refractive to transcriptional activation (Chen et al. 1999).

Once in the nucleus, β-catenin is thought to convert the TCF repressor complex into a transcriptional activator complex. This may occur through displacement of Groucho from TCF/LEF and recruitment of the histone acetylase CBP/p300 (cyclic AMP response element-binding protein). CBP may bind to the β-catenin/TCF complex as a coactivator (Hecht et al. 2000, Takemaru & Moon 2000), a hypothesis that remains to be tested directly. Another activator, Brg-1, is a component of the SWI/SNF (switching-defective and sucrose nonfermenting) chromatin remodeling complex which, with CBP, may induce chromatin remodeling that favors target gene transcription (Barker et al. 2001).

Further interactions between the TCF-β-catenin complex and chromatin could be mediated by Legless (Bcl9) and Pygopos (Kramps et al. 2002, Parker et al. 2002, Thompson et al. 2002). Mutations in either of these genes result in *wingless*-like phenotypes in *Drosophila*, and both genes promote Wnt signaling in mammalian cell culture experiments (Thompson et al. 2002).

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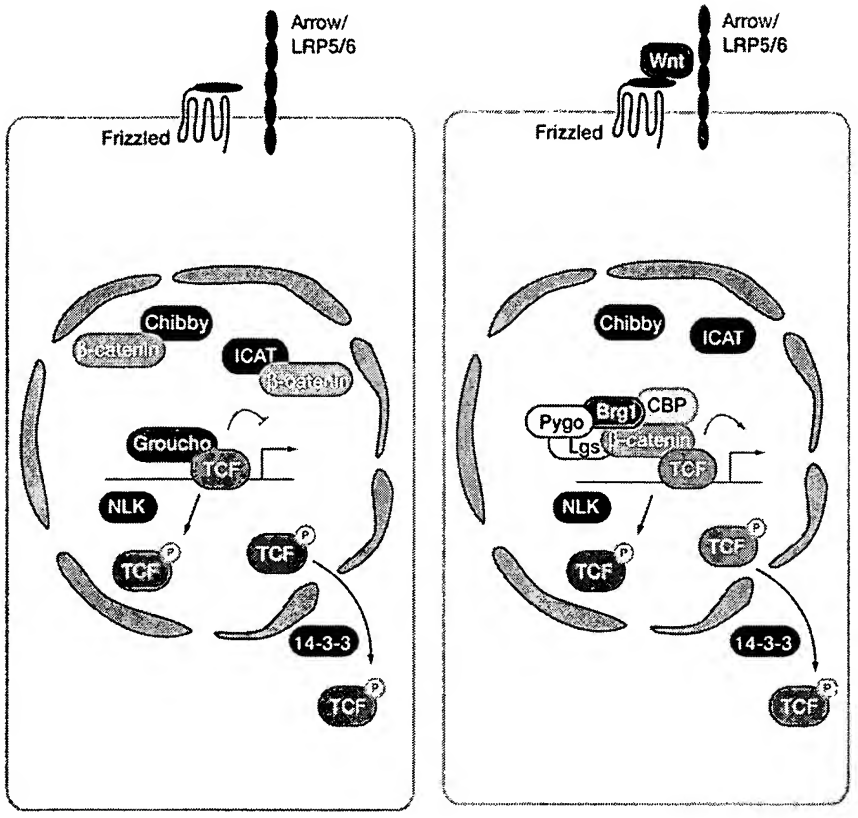

Figure 4 Nuclear factors in Wnt signaling. The interaction between Groucho and TCF is thought to down-regulate transcriptional activation (*left panel*). β-catenin is also negatively regulated by binding to Chibby and Inhibitor of β-catenin and TCF (ICAT). TCF activity in the nucleus can be modulated by phosphorylation by Nemo-like kinase (NLK), and in *C. elegans*, a 14-3-3-like protein has been shown to facilitate nuclear export of TCF (*thin arrow*). β-catenin interferes with the interaction between TCF and Groucho,, and together with TCF, activates gene expression. β-catenin also binds to other components such as Legless (Lgs), Pygopus (Pygo), CREB-binding protein (CBP), and Brg1. Negative regulators are shown in black. Positively acting components are outlined in color.

Wnt signaling events in the nucleus are controlled by a number of protein partners. For example, the protein Chibby is a nuclear antagonist that binds to the C terminus of β-catenin (Takemaru et al. 2003). Another β-catenin-binding protein, ICAT (Tago et al. 2000), not only blocks the binding of β-catenin to TCF (Tago et al. 2000) but also leads to dissociation of complexes between β-catenin, LEF, and CBP/p300 (Daniels & Weis 2002, Graham et al. 2002). TCF is also subject

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to regulation, as it can be phosphorylated by the mitogen-activated protein (MAP) kinase–related protein kinase NLK/Nemo (Ishitani et al. 1999). NLK/Nemo itself is activated by the mitogen-activated protein (MAP) kinase kinase, TAK1 (Ishitani et al. 1999). The phosphorylation of TCF/LEF by activated Nemo is thought to diminish the DNA-binding affinity of the β-catenin/TCF/LEF complex, thereby affecting transcriptional regulation of Wnt target genes (Ishitani et al. 1999, 2003). Another consequence of TCF phosphorylation, at least in *C. elegans*, is export of TCF from the nucleus (Meneghini et al. 1999), which is carried out by a 14-3-3 protein, Par5 (Lo et al. 2004). The ability of LEF/TCF to interact with DNA and its other partners is therefore highly regulated and likely plays critical roles in the modulation of Wnt target gene expression. Recently, it was shown that β-catenin can interact with other binding partners in the nucleus, such as Pitx2. β-catenin can convert Pitx2 from a transcriptional repressor into an activator (Kioussi et al. 2002), similar to its interaction with LEF1/TCF. The presence of additional β-catenin-binding partners adds another layer of complexity to the regulation of gene expression by nuclear β-catenin.

WNT TARGET GENES AND FEED BACK LOOPS

Mutant analysis of Wnt genes has shed light on the range of biological processes that Wnts control. In particular, *wingless* in *Drosophila* is involved in numerous developmental events that include embryonic and larval patterning (Cadigan & Nusse 1997) and synaptic differentiation (Packard et al. 2002). In vertebrate development, loss of a single Wnt gene can produce dramatic phenotypes that range from embryonic lethality and CNS abnormalities to kidney and limb defects (Table 1). These diverse phenotypes indicate that the Wnt pathway has distinct transcriptional outputs. In many cases, the cell, rather than the signal, determines the nature of the response, and up- or down-regulation of Wnt target genes is cell-type specific. In other cases, however, the same target genes can be induced in multiple cell and tissue types. Whether these are universal targets of Wnt signaling remains to be shown.

Interestingly, there may be some themes in the types of target genes that are induced by Wnts. Wnt signaling can promote the expression of Wnt pathway components (Table 2). Whether these genes are direct Wnt targets is not known in all cases, but this finding indicates that feedback control is a key feature of Wnt signaling regulation. One class of targets that respond to Wnt signaling is the Frizzleds (Cadigan et al. 1998, Muller et al. 1999, Sato et al. 1999, Willert et al. 2002). Dfz2 in *Drosophila* is down-regulated by *wg* wherever Wg is active, a process that may function to limit the levels of Wnt signaling within the Dfz2-expressing cells. In addition, by reducing the levels of a high-affinity receptor that might otherwise limit Wg distribution, Wg may be allowed to diffuse over longer distances (Cadigan et al. 1998). The levels of LRP and HSPG are also controlled by Wg signaling, providing further fine-tuning of Wg activity at the cell surface

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TABLE 1 Wnt mutant phenotypes in the mouse

Gene	Knockout (KO) phenotypes or other functions	Redundancies/ similarities with other KO	References
Wnt1	Deficiency in neural crest derivatives, reduction in dorsolateral neural precursors in the neural tube (with Wnt3A KO) Decrease in thymocyte number (with Wnt-4 KO)	Redundant with Wnt3a and Wnt4; Similar to TCF1	(Ikeya et al. 1997, Mulroy et al. 2002)
Wnt3	Early gastrulation defect, Perturbations in establishment and maintenance of the apical ectodermal ridge (AER) in the limb	In limbs, similar to loss of β-catenin	(Barrow et al. 2003, P. Liu et al. 1999)
Wnt3a	Paraxial mesoderm defects, tailbud defects, deficiency in neural crest derivatives, reduction in dorsolateral neural precursors in the neural tube (with Wnt1 KO) Loss of hippocampus Somitogenesis defects	Redundant with Wnt1, Similar to LEF1/TCF1	(Aulehla et al. 2003; Galceran et al. 1999, 2000; Ikeya et al. 1997; Lee et al. 2000; Yoshikawa et al. 1997)
Wnt4	Defects in female development; absence Mullerian duct, defects in adrenal gland development Decrease in thymocyte number (with Wnt1 KO)	Wnt1	(Heikkila et al. 2002, Mulroy et al. 2002, Vainio et al. 1999)
Wnt5a	Truncated limbs and AP axis Defects in distal lung morphogenesis Chondrocyte differentiation defects, perturbed longitudinal skeletal outgrowth Inhibits B cell proliferation, produces myeloid leukemias and B-cell lymphomas in heterozygotes		(Li et al. 2002, Liang et al. 2003, Yamaguchi et al. 1999, Y. Yang et al. 2003)
Wnt7a	Female infertility; in males, Mullerian duct regression fails Delayed maturation of synapses in cerebellum		(Hall et al. 2000, Parr & McMahon 1998)
Wnt7b	Placental development defects Respiratory failure; defects in early mesenchymal proliferation leading to lung hypoplasia		(Parr et al. 2001, Shu et al. 2002)
Wnt11	Ureteric branching defects and kidney hypoplasia		(Majumdar et al. 2003)

TABLE 2 Wnt signaling components as Wnt pathway targets

Target gene	Effect of Wnt signal on target gene expression	Effect of changes in target gene expression on Wnt pathway	Target gene interacts with	Reference
Fz	Down	Inactivate	Wnt	(Muller et al. 1999)
Dfz2	Down	Inactivate	Wnt	(Cadigan et al. 1998)
Dfz3	Up	Activate	Wnt	(Sato et al. 1999)
Fz7	Up		Wnt	(Willert et al. 2002)
Arrow/LRP	Down	Inactivate	Wnt	(Wehrli et al. 2000)
Dally (HSPG)	Down		Wnt	(Baeg et al. 2001)
Wingful/notum	Up	Inactivate	HSPG?	(Giraldez et al. 2002)
naked	Up	Inactivate	Dsh	(Rousset et al. 2001)
Axin2	Up	Inactivate	β-catenin	(Jho et al. 2002)
β-TCRP	Up	Inactivate	β-catenin	(Spiegelman et al. 2000)
TCF1 (dn)	Up	Inactivate	TCF	(Roose et al. 1999)
LEF1	Down	Activate	β-catenin	(Hovanes et al. 2001)
Nemo	Up	Inactivate (*Drosophila*) Activate (*Zebrafish*)	β-catenin/ LEF/TCF	(Zeng & Verheyen 2004, Thorpe & Moon 2004)

(Baeg et al. 2001, Wehrli et al. 2000). Two cytoplasmic negative regulators are also induced by Wnt signals. The *naked cuticle* (*naked*) gene is an EF-hand-containing protein that can bind directly to Dsh and inhibit Wnt signaling in both *Drosophila* (Rousset et al. 2001) and vertebrates (Wharton et al. 2001). The *Axin2*[gene is also a direct Wnt target that is expressed in many sites where Wnt signaling is known to occur (Aulehla et al. 2003, Jho et al. 2002). TCF and LEF are transcriptionally responsive to Wnt signaling. In colorectal cancer cells, loss of APC up-regulates LEF1, which may promote increased mis-regulation of Wnt target genes (Hovanes et al. 2001). APC mutations also up-regulate a splice-variant of TCF1 that lacks an N-terminal β-catenin-binding site (Roose et al. 1999). This dominant-negative TCF1 is thought to dampen Wnt signaling and reduce the severity of perturbations that result from loss of β-catenin or APC. Therefore, although APC mutations ultimately induce cancerous lesions in the colon, the induction of both TCF1 and LEF1 expression reveals an exquisite ability of colon cells to modulate Wnt signaling levels through feed-back regulation.

Cell proliferation is commonly regulated by Wnt signaling, and Wnt knockout phenotypes can often be explained by a loss of cell proliferation. For example, limb outgrowth fails in limb buds lacking Wnt5A (Yamaguchi et al. 1999), and expansion of the CNS fails in Wnt1 mutants (Megason & McMahon 2002). A mitogenic effect of *wingless* has also been reported for the *Drosophila* wing imaginal disc (Giraldez & Cohen 2003). The loss of particular cells or tissues in Wnt mutants has often been interpreted as stemming from perturbations in cell fate specification,

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but an alternative interpretation may be that in some cases, progenitor cells fail to expand. A general function of Wnt signaling during development may therefore be to regulate the cell proliferation by direct induction of cell cycle regulators. Consistent with this, myc and cyclinD1 are direct Wnt signaling targets in colon cancer cells (He et al. 1998, Shtutman et al. 1999, Tetsu & McCormick 1999).

A recent study in the skin (Jamora et al. 2003) has raised the intriguing possibility that Wnt signaling might also generally regulate cell adhesion, although this must be tested further. Formation of an epithelial bud during hair follicle development requires the repression of E-cadherin transcription. Inputs from both Wnt, which stabilizes β-catenin, and from the BMP inhibitor Noggin, which induces Lef1 expression, directly repress the E-cadherin promoter. If the Wnts can regulate cell-cell adhesion molecules at a transcriptional level, then Wnt signaling may integrate cell fate specification and differentiation with cell behavior changes. It will be interesting to determine whether Wnts regulate cadherin transcription in places such as the teeth and mammary gland where morphogenetic movements similar to those accompanying epithelial bud formation occur (van Genderen et al. 1994). Several components of the Wnt pathway such as β-catenin and APC are also multifunctional, participating in cell-cell adhesion and cytoskeletal rearrangements (reviewed in Bienz 2002, Gumbiner 2000). Therefore, the possibility that Wnt signaling, through changes in β-catenin levels, directly impinges on cell adhesion or cell behavior is tantalizing. and provides exciting avenues for further research. For a discussion of current data that examines connections between cell adhesion and Wnt signaling, we direct the reader to Nelson & Nusse (2004).

WNT PHENOTYPES: REDUNDANCY AND SPECIFICITY

A common approach to understanding the function of a particular gene in a tissue or developmental process is to examine its knockout phenotype. In some cases, the expression patterns and mutant phenotypes correlate closely and clearly demonstrate the requirement for that particular Wnt in a specific developmental event. Wnt3, for example, is expressed in the primitive streak in the early mouse embryo, and Wnt3 mutants display gastrulation defects (P. Liu et al. 1999). There are several cases, however, where mutant phenotypes were not fully revealed until multiple Wnts were removed. One classic example is the Wnt1/Wnt3a double-knockout, which demonstrates a requirement for Wnt signaling in a wider region of the CNS than when only the Wnt1 or Wnt3a gene is eliminated (Table 1) (Ikeya et al. 1997). This is also observed with downstream Wnt pathway components; mutants of Lef1 and TCF1 exhibit defects similar to Wnt3a mutants but only when both Lef1 and TCF1 are missing (Table 3). These examples illustrate that genetic redundancy between Wnt signaling components is likely to greatly influence our ability to discern and interpret Wnt mutant phenotypes.

The analysis of Fz mutants has largely failed to reveal specific Wnt/Fz pairs that interact during development. An exception is Frizzled 4, which affects axonal

TABLE 3 TCF mutant phenotypes in vertebrates

Gene	Knockout (KO) phenotypes or other functions	References
Tcf1 (official name *Tcf7*)	Thymocyte differentiation defects Defects in limb bud development (with Lef1 KO) Mammary and gut tumors, accelerated by loss of Min/APC	(Galceran et al. 1999, Roose et al. 1999, Verbeek et al. 1995)
Tcf3 (official name *Tcf7L1*) *headless* in Zebrafish	Expanded axial mesoderm in mice, anterior defects in Zebrafish	(Kim et al. 2000, Merrill et al. 2004)
Tcf4 (official name *Tcf7L2*)	Absence of epithelial stem cells in small intestine	(Korinek et al. 1998)
Lef1	Defects in limb bud development (with Tcf1 KO) Defects in pro-B cell proliferation and survival	(Galceran et al. 1999, Reya et al. 2000)

TABLE 4 Frizzled phenotypes in mammals

Gene	Knockout (KO) phenotypes or other functions	References
Fz3 (*Mfz3*)	Defect in fiber tracts in the rostral CNS Perturbed anterior-posterior guidance of commissural axons	(Lyuksyutova et al. 2003, Wang et al. 2002)
Fz4 (*Mfz4*)	Cerebellar, auditory, and esophageal defects In humans, retinal angiogenesis in familial exudative vitreoretinopathy (FEVR)	(Wang et al. 2001, Robitaille et al. 2002, Xu et al. 2004, Toomes et al. 2004)
Fz5 (*Mfz5*)	Essential for yolk sac and placental angiogenesis	(Ishikawa et al. 2001)
Fz6 (*Mfz6*)	Hair patterning defects	(Guo et al. 2004)

guidance in the neural tube by a process that may involve Wnt4 (Lyuksyutova et al. 2003) (Table 4). The lack of a one-to-one correspondence between individual Wnt and Fz mutant phenotypes suggests that a single Frizzled might be activated by multiple Wnts or that a given Wnt might bind multiple Frizzleds. In *Drosophila*, Wg binds to both Fz and Dfz2 and a cuticle patterning defect is observed only when both receptors are mutant (Bhanot et al. 1999, Chen & Struhl 1999, Kennerdell & Carthew 1998, Rulifson et al. 2000). This is a relatively simple example where signaling is mediated primarily by one Wnt and there is genetic redundancy between only two Fzs. In vertebrates, where Wnt and Fz expression patterns may be more elaborate, and where Frizzled may even interact with other ligands such as

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Norrin (Guo et al. 2004), the overlapping interactions and relationships between Wnts, other Frizzled binding partners, and Fzs are likely to be far more complex.

In a given cell or tissue, only a subset of Wnts can stimulate the canonical signaling pathway (for examples, see Kispert et al. 1998, Shimizu et al. 1997, Torres et al. 1996). These data likely reflect the ability of different Wnts to bind to the particular receptors that exist on the surfaces of the responding cells. Studies that measure Wnt-Fz binding affinities are only just beginning (Hsieh et al. 1999b, Wu & Nusse 2002). Not much is known about specificity between ligands and receptors in vertebrates, but in *Drosophila*, the affinity between Wingless and its receptors, Fz and Dfz2, is high (Wu & Nusse 2002). As more binding studies are performed, they will provide valuable tools for elucidating physiological interactions between Wnts and Fzs.

WNT SIGNALING IN CANCER AND HUMAN DISEASE

Given the diverse phenotypes produced by Wnt knockouts in mice, it is not surprising that loss of Wnts in humans has dire consequences as well. Recently, Tetra-amelia, a rare human genetic disorder characterized by absence of limbs, has been proposed to result from WNT3 loss-of- function mutations (Niemann et al. 2004).

In adults, mis-regulation of the Wnt pathway also leads to a variety of abnormalities and degenerative diseases (Table 5). An LRP mutation has been identified that causes increased bone density at defined locations such as the jaw and palate (Boyden et al. 2002, Little et al. 2002). The mutation is a single amino-acid substitution that makes LRP5 insensitive to Dkk-mediated Wnt pathway inhibition, indicating that the phenotype results from overactive Wnt signaling in the bone (Boyden et al. 2002). In a different study, mutations in LRP5 were correlated with

TABLE 5 Human genetic diseases and Wnt signaling components

Gene	Disease	References
WNT3	Tetra-amelia	(Niemann et al. 2004)
LRP5	Bone density defects Vascular defects in the eye (osteoperosis-pseudoglioma syndrome; OPPG), familial exudative vitreoretinopathy; FEVR)	(Boyden et al. 2002, Gong et al. 2001, Little et al. 2002, Toomes et al. 2004)
FZD4	Retinal angiogenesis defects (familial exudative vitreoretinopathy; FEVR)	(Robitaille et al. 2002 Xu et al. 2004, Toomes et al. 2004)
Axin2	Tooth agenesis Predisposition to colorectal cancer	(Lammi et al. 2004)
APC	Polyposis coli, colon cancer	(Kinzler et al. 1991, Nishisho et al. 1991)

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decreased bone mass (Gong et al. 2001). In this case, frame shift and missense mutations were thought to create loss-of-function LRP5 mutations. These data indicate that Wnt signaling mediated by LRP5 is required for maintenance of normal bone density.

LRP5 mutations (Gong et al. 2001) can also be accompanied by vasculature defects in the eye (osteoperosis-pseudoglioma syndrome or OPPG). In addition, a hereditary disorder, called familial exudative vitreopathy (FEVR), is caused by mutations in both LRP5 and the Fz4 receptor, which results in defective vasculogenesis in the peripheral retina (Toomes et al. 2004, Robitaille et al. 2002). The Fz4 mutation is located in the seventh transmembrane domain, and the LRP5 mutations all create prematurely terminated proteins, suggesting that FEVR results from loss of Fz/LRP signaling. More recently, Norrin, a protein that bears no ressemblance to Wnts, has been identified as the ligand for the Fz4/LRP receptor complex (Xu et al. 2004). Signaling by functional Norrin/Fz/LRP complexes is therefore crucial for proper vasculogenesis and its maintenance in at least some parts of the body.

Mutations in intracellular Wnt pathway components also produce dramatic defects. A nonsense mutation in Axin2 has been shown to produce severe tooth agenesis, or oligodontia, a condition in which multiple permanent teeth are missing (Lammi et al. 2004).

Mutations that promote constitutive activation of the Wnt signaling pathway lead to cancer. In addition to tooth defects, individuals with Axin2 mutations display a prediposition to colon cancer (Lammi et al. 2004). Moreover, the best-known example of a disease involving a Wnt pathway mutation that produces tumors is familial adenomatous polyposis (FAP), an autosomal, dominantly inherited disease in which patients display hundreds or thousands of polyps in the colon and rectum. This disease is caused most frequently by truncations in APC (Kinzler et al. 1991, Nishisho et al. 1991), which promote aberrant activation of the Wnt pathway leading to adenomatous lesions owing to increased cell proliferation. Mutations in β-catenin and APC have also been found in sporadic colon cancers and a large variety of other tumor types (reviewed in Giles et al. 2003). Loss-of-function mutations in Axin have been found in hepatocellular carcinomas (Satoh et al. 2000). These examples demonstrate that the uncoupling of normal β-catenin regulation from Wnt signaling control is an important event in the genesis of many cancers.

It has become increasingly common to view cancer as a stem cell disease (see Taipale & Beachy 2001). In the colon, loss of TCF4 or Dkk overexpression promotes loss of stem cells in the colon crypt, indicating that Wnt signaling is required for maintenance of the stem cell compartment (Korinek et al. 1998, Kuhnert et al. 2004, Pinto et al. 2003). A more extensive discussion of Wnt signaling and colon stem cell control is presented elsewhere in this volume (see Sancho et al. 2004. Wnt signaling may therefore be a fundamental regulator of stem cell choices to proliferate or self-renew. Consistent with this idea, Wnt3a promotes self-renewal of hematopoietic stem cells in vitro (Willert et al. 2003). The use of soluble Wnts to control the proliferation and/or maintenance of stem cells may offer powerful therapeutic reagents for the in vitro manipulation of stem cells and their reintroduction into diseased tissues.

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EVOLUTIONARY ORIGIN WNT SIGNALING

Is it possible to trace the evolutionary origins of Wnt signaling? With the completion of several animal genomes and partial sequence information on other organisms, these questions can now be addressed in a systematic manner. The finished genomes of some mammals and invertebrate organisms has led to catalogues of 19 Wnt genes in the human and the mouse, 7 in *Drosophila*, and 5 in *C. elegans*. Between *Drosophila* and mammals, there is fairly extensive conservation of Wnt genes, so that orthologs can readily be recognized. These orthologous relationships suggest similar biological or biochemical activities, the orthologs Wnt1 and wingless, for example, both regulate the expression of their target gene, *engrailed* (Danielian & McMahon 1996). Between *Drosophila* and vertebrates, there is also conservation of clusters of genes. This indicates that there was a common ancestral cluster of Wnt genes containing *WNT1*, *WNT6*, and *WNT10* that predated the last common ancestor of arthropods and deuterostomes (Nusse 2001, Prud'homme et al. 2002).

Members of the Cnidaria, which are primitive diploblasts, contain a bona fide Wnt and a complete set of Wnt pathway genes (Hobmayer et al. 2000). In another member of the Cnidaria family, the Anemone *Nematostella*, a β-catenin homolog has been shown to be involved in Axis specification and the formation of endoderm (Wikramanayake et al. 2003). Sponges have a Fz gene (Adell et al. 2003), providing another striking example of the conservation of Wnt signaling pathway components throughout evolution.

In yet other primitive organisms, components of the pathway are present, but not necessarily regulated by a Wnt signal. *Dictyostelium* has a vestige of a Wnt pathway, as a gene called *aardvark* is not only a β-catenin homolog (Grimson et al. 2000) but is also regulated by GSK-3 phosphorylation (Grimson et al. 2000). However, there is no evidence for Wnt-like genes in this organism, and although several seven transmembrane molecules act as receptors for cyclic AMP and regulate GSK activity (Plyte et al. 1999), there is no significant homology between those receptors and the Frizzleds. There are clearly recognizable homologs of β-catenin in plants (Amador et al. 2001). It is possible, therefore, that an ancient β-catenin-based mechanism existed prior to the evolution of animals. By adding Wnt and Frizzleds, β-catenin activity became subject to control from other cells, a quintessential aspect of organized multicellular life.

CONCLUDING REMARKS

The past few years have been accompanied by an explosion of data that implicates Wnt signaling in development and in adult tissue maintenance. Given the number of Wnt genes and their widely ranging functions, a large fraction of developmental decisions during the lifetime of an animal may be influenced by a Wnt signal. It is not surprising that mis-regulation of such an important pathway leads to disease, and the role of Wnt signaling in cancer is now well established. Whereas we have

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an increasingly detailed picture of Wnt signaling as a complex, tightly regulated pathway with many functions, the mechanisms of several outstanding events during Wnt signal transduction still need to be resolved. These include understanding how Wnts are secreted and presented to cells, how Wnt binding to the Fz/LRP complex transduces a signal to Dsh, how proteins within the β-catenin degradation complex are regulated, and how inputs from positive and negative regulators are integrated within the nucleus to effect transcription. In addition, translating our knowledge of Wnt signaling into some form of intervention for disease is a formidable but important challenge. Fortunately, powerful new experimental methods and reagents for the study of Wnt signaling have recently become available. These include purified Wnt ligands (Willert et al. 2003), small molecules that either activate (Meijer et al. 2003) or inhibit Wnt signaling (Lepourcelet et al. 2004), and RNAi screens for components of the Wnt signaling pathway (Boutros et al. 2004, Lum et al. 2003). The quest for additional tools to manipulate this pathway, to a large extent driven by the potential use of these reagents in managing disease, will lead to further insights into the complexity and intricacy of the Wnt signal transduction cascade.

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EXHIBIT 2

Protein family review

The Wnts

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Summary

The *Wnt* genes encode a large family of secreted protein growth factors that have been identified in animals from hydra to humans. In humans, 19 WNT proteins have been identified that share 27% to 83% amino-acid sequence identity and a conserved pattern of 23 or 24 cysteine residues. *Wnt* genes are highly conserved between vertebrate species sharing overall sequence identity and gene structure, and are slightly less conserved between vertebrates and invertebrates. During development, Wnts have diverse roles in governing cell fate, proliferation, migration, polarity, and death. In adults, Wnts function in homeostasis, and inappropriate activation of the Wnt pathway is implicated in a variety of cancers.

Gene organization and evolutionary history

Gene organization

In humans, 19 *WNT* genes have been identified and the chromosomal locations of each is known (see Table 1) [1-6]. Several human *WNT* genes are located very close to each other in the genome [7,8]; these include *WNT6* and *WNT10a*, which are located immediately adjacent to one another on chromosome 2 (about 6.4 kilobases (kb) apart), and *WNT1* and *WNT10b*, which are located adjacent to each other on chromosome 12 (about 8.1 kb apart). *WNT6* and *WNT10a* are transcribed in opposite directions, whereas *WNT1* and *WNT10b* are expressed from the same strand of DNA. Several additional pairs of *WNT* genes are also clustered within the human genome, including *WNT2* and *WNT16* (about 4 megabases (Mb) apart), *WNT3a* and *WNT14* (about 250 kb apart), and *WNT3* and *WNT15*. In the mouse, there are at least 18 *Wnt* genes and the locations of all but two of them have been determined [1-3,5,6]. As in humans, the mouse *Wnt1/Wnt10b*, *Wnt6/Wnt10a*, and *Wnt3/Wnt15* gene pairs are each located on the same chromosomes, and in the case of the *Wnt1/Wnt10b* and *Wnt6/Wnt10a* pairs the close proximity of these genes has been conserved from mouse to human. Interestingly, in the *Drosophila* genome, the paralogous genes *wingless* (*wg*), *DWnt6* and *DWnt10*, are located immediately adjacent to one another on the second chromosome and are all transcribed in the same orientation. Thus, it is possible that there was an ancient cluster of *Wnt* genes consisting of *Wnt1*, *Wnt6* and *Wnt10* in a common ancestor of vertebrates and arthropods. In vertebrates, this cluster may have been duplicated with subsequent loss of *Wnt1* from one cluster and *Wnt6* from the other.

The majority of human *WNT* genes contain four coding exons, with exon 1 containing the initiation methionine (Figure 1a) [8]. *WNT* genes that differ from this pattern include *WNT14*, with three exons, *WNT2*, *WNT5b*, and *WNT11*, with five exons, and *WNT8b* with six exons. Several *WNTs* - *WNT2b/13*, *WNT8a/d*, and *WNT16* - have alternative amino or carboxyl termini, which result from the use of alternative 5′ or 3′ exons.

Evolutionary history

The deduced evolutionary relationships of 18 of the 19 known human *WNT* genes are shown in Figure 2. The majority of Wnt proteins share about 35% amino-acid sequence identity, although members of a subgroup (those with the same numeral, such as *WNT3* and *WNT3a*) share increased sequence identity (from 58% to 83%) and some overlapping sites of expression. Members of subgroups are not closely linked within the genome, however, suggesting that they were generated by gene-translocation or genome-duplication events, not by local duplication events.

Table 1

Chromosomal locations of *WNT* genes in human and mouse

Human		Mouse		References	Accession numbers†	
Gene	Location	Gene	Location*		Human	Mouse
WNT1	12q13	*Wnt1*	15	[87-91]	X03072	K02593
WNT2	7q31	*Wnt2*	6 (4.2 cM)	[92,93]	X07876	AK012093
WNT2b/13	1p13	*Wnt2b/13*	3 (49.0 cM)	[94-96]	XM052111, XM052112	AF070988
WNT3	17q21	*Wnt3*	11 (63.0 cM)	[97-100]	AY009397	M32502
WNT3a	1q42.13	*Wnt3a*	11 (32.0 cM)	[101-103]	AB060284	X56842
WNT4	1p35	*Wnt4*	4	[100,104]	AY009398	M89797
WNT5a	3p14-p21	*Wnt5a*	14 (14.8 cM)	[104-106]	L20861	M89798
WNT5b	12p13.3	*Wnt5b*	6 (56.2 cM)	[104,107]	AB060966	M89799
WNT6	2q35	*Wnt6*	1	[104,108,109]	AY009401	M89800
WNT7a	3p25	*Wnt7a*	6 (39.5 cM)	[104,106,110,111]	D83175	M89801
WNT7b	22q13.3	*Wnt7b*	15 (46.9 cM)	[100,104,112,113]	AB062766	M89802
WNT8a/d	5q31	*Wnt8a*		[114,115]	AB057725, AY009402	Z68889
WNT8b	10q24	*Wnt8b*	19 (43.0 cM)	[116-118]	Y11094	AF130349
WNT10a	2q35	*Wnt10a*	1	[109,119]	AB059569	U61969
WNT10b/12	12q13.1	*Wnt10b*	15 (56.8 cM)	[106,119-124]	U81787	U61970
WNT11	11q13.5	*Wnt11*	7	[106,125]	Y12692	X70800
WNT14	1q42	-		[103,126]	AB060283	
WNT15	17q21	*Wnt15*	11	[126]	AF028703	AF031169
WNT16	7q31	*Wnt16*		[127,128]	XM031374, XM004884	AF172064

*Locations of mouse genes give the chromosome and the distance in centimorgans (cM) from the telomere. †Accession numbers are for GenBank [3].

Wnt genes have been identified in vertebrates and invertebrates, but appear to be absent from plants, unicellular eukaryotes such as *Saccharomyces cerevisiae* and from prokaryotes. To date, in vertebrates, 16 *Wnt* genes have been identified in *Xenopus*, 11 in chick, and 12 in zebrafish [5]; in invertebrates, *Drosophila* has seven *Wnt* genes, *Caenorhabditis elegans* five and *Hydra* at least one [5]. The apparent evolutionary relationships between selected invertebrate and vertebrate *Wnt* genes are shown in Figure 2b. In vertebrates, the orthologs in different species are highly similar in sequence. For example, human WNT1 and mouse Wnt1 are 98% identical, and human WNT5a and *Xenopus* Wnt5a are 84% identical at the amino-acid level. Phylogenetic analyses of vertebrate and invertebrate Wnts demonstrate orthologous relationships between several human and *Drosophila Wnts* (Figure 2b). The sequence identity between orthologous proteins in humans and flies ranges from 21% between human WNT8a/d and *Drosophila* DWnt8 to 42% sequence identity between human WNT1 and *Drosophila* Wingless (Wg). The evolutionary relationship between the five *C. elegans Wnt* genes and human *WNT* genes is less apparent, making it difficult to determine which *C. elegans Wnt* genes may have orthologs in the human genome.

Characteristic structural features

Human WNT proteins are all very similar in size, ranging in molecular weight from 39 kDa (WNT7a) to 46 kDa (WNT10a) [3]. *Drosophila* Wnt proteins are also similar to this, with the exception of Wg, which is approximately 54 kDa and has an internal insert not found in vertebrate Wnts, and DWnt3/5, which is about 112 kDa [3]. Very little is known about the structure of Wnt proteins, as they are notoriously insoluble, but all have 23 or 24 cysteine residues, the spacing of which is highly conserved (Figure 1b), suggesting that Wnt protein folding may depend on the formation of multiple intramolecular disulfide bonds. Analysis of the signaling activities of chimeric Wnt proteins has shown that the carboxy-terminal region of Wnt proteins may play a role in determining the specificity of responses to different Wnts [9]. Furthermore, deletion mutants lacking the carboxy-terminal third of a Wnt protein can act as

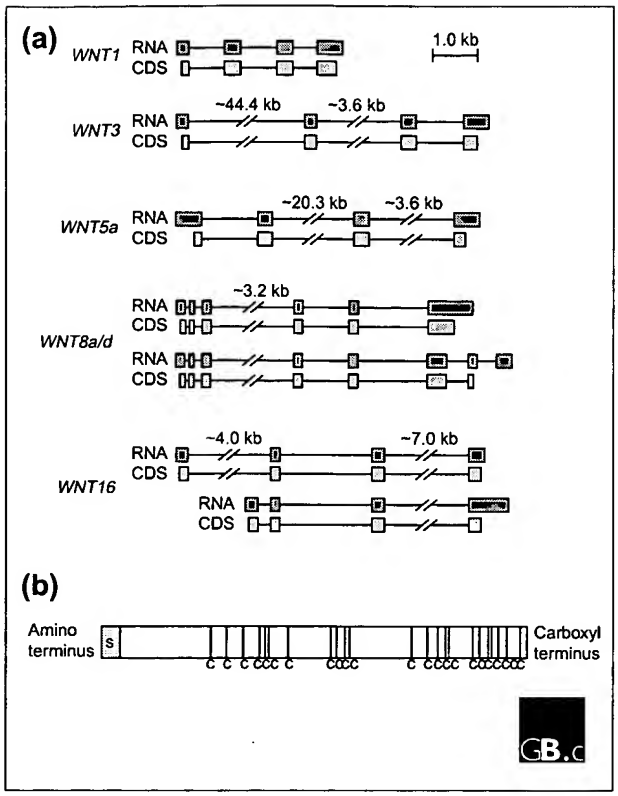

Figure 1
(a) Structures of selected members of the human *WNT* gene family. Exons are shown as boxes and introns as lines. For each gene, 'RNA' represents the portion of the gene that is transcribed and 'CDS' represents the portion that encodes protein. *WNT8a/d* is an example of a gene with 3' alternative splicing and *WNT16* is an example of a gene with alternatively used 5' exons. **(b)** Structural features of the Wnt protein. The amino terminus contains a signal sequence (S). All Wnts contain 23 or 24 conserved cysteine residues (C) with similar spacing, suggesting that the folding of Wnt proteins depends on the formation of multiple intramolecular disulfide bonds.

dominant-negatives in a cell-non-autonomous manner [10], suggesting that the amino-terminal region may mediate interactions with Wnt receptors but requires the carboxyl terminus to activate these receptors.

Localization and function

Post-translational modifications and secretion

Wnt proteins have an amino-terminal signal sequence, can act in a cell non-autonomous manner, and are present in the secretory pathway, indicating that they are secreted proteins [11]. In addition, genetic analyses of Wg signaling in *Drosophila* uncovered mutations in the *porcupine* gene that show a lack of Wnt activity due to the retention of Wg protein in the endoplasmic reticulum [12-14]. The *porcupine* gene is predicted to encode a protein with eight transmembrane domains and has a perinuclear localization in transfected cells [14]; overexpression of *porcupine* does not increase levels of secreted Wg but does change the pattern of Wg glycosylation [14]. In worms, *mom-1* encodes a *porcupine* homolog and, when mutated, phenocopies mutants of *mom-2*, which encodes a Wnt, suggesting that the function of *porcupine* is conserved [15,16]. Although size chromatography suggests that Wg is secreted as a multimer, it remains unclear whether Wnt proteins in general are secreted as monomers, oligomers, or as part of a multi-protein complex [17]. Wnt proteins are glycosylated, but mutation of some or all of the predicted glycosylation sites in mouse Wnt1 does not abolish its activity in cultured cells [18]; these modifications may thus be unimportant for Wnt function.

Subcellular localization

Once secreted, Wnt proteins associate with glycosaminoglycans in the extracellular matrix and are bound tightly to the cell surface [19,20]. Although Wnts are found in tight association with the plasma membrane, it is possible to collect active Wnt from the medium of cultured cells [21,22]. Beyond this information, the localization of Wnt proteins in vertebrates is poorly understood. Examination of the localization of Wg in *Drosophila*, however, has provided critical insights into the subcellular distribution of Wnt proteins and the importance of this distribution for signaling activity. In the embryonic epidermis, Wg is found inside cells that secrete Wg and in association with the plasma membrane of secreting cells and non-secreting cells several cell diameters from the Wg source [23]. Wg is also prevalent in vesicles and multi-vesicular bodies of non-Wg-producing cells anterior to the source of Wg, suggesting that Wg is endocytosed [23,24]. This idea is supported by examination of *shibire* embryos, which have a mutation in dynamin, a critical component of the endocytic machinery; these mutants have defects in Wg distribution, and Wg signaling activity is compromised [25]. Similarly, expression of a dominant-negative form of *shibire* also reduces Wg activity [26]. Endocytosis may also help to limit the distribution of Wg signal. In contrast to cells anterior to the Wg source, cells posterior to Wg-producing cells have much lower levels of Wg in endocytic vesicles, and this asymmetry in distribution mirrors the observation that Wg acts over a much shorter range towards the posterior than towards the anterior. This difference in Wg distribution appears to be due to rapid degradation of endocytosed Wg in posterior cells [27]. The spatially restricted pattern of Wg degradation is regulated by signals through the epidermal growth factor (EGF) receptor that hasten the destruction of Wg in posterior cells [27].

Association of Wg with specific membrane microdomains also appears to play a role in controlling the distribution of Wg signals during *Drosophila* development. In imaginal discs, Wg is found in specialized membrane vesicles called argosomes, which are thought to be derived from lipid raft microdomains [28]. Incorporation of Wg into argosomes requires heparan sulfate proteoglycans, suggesting that

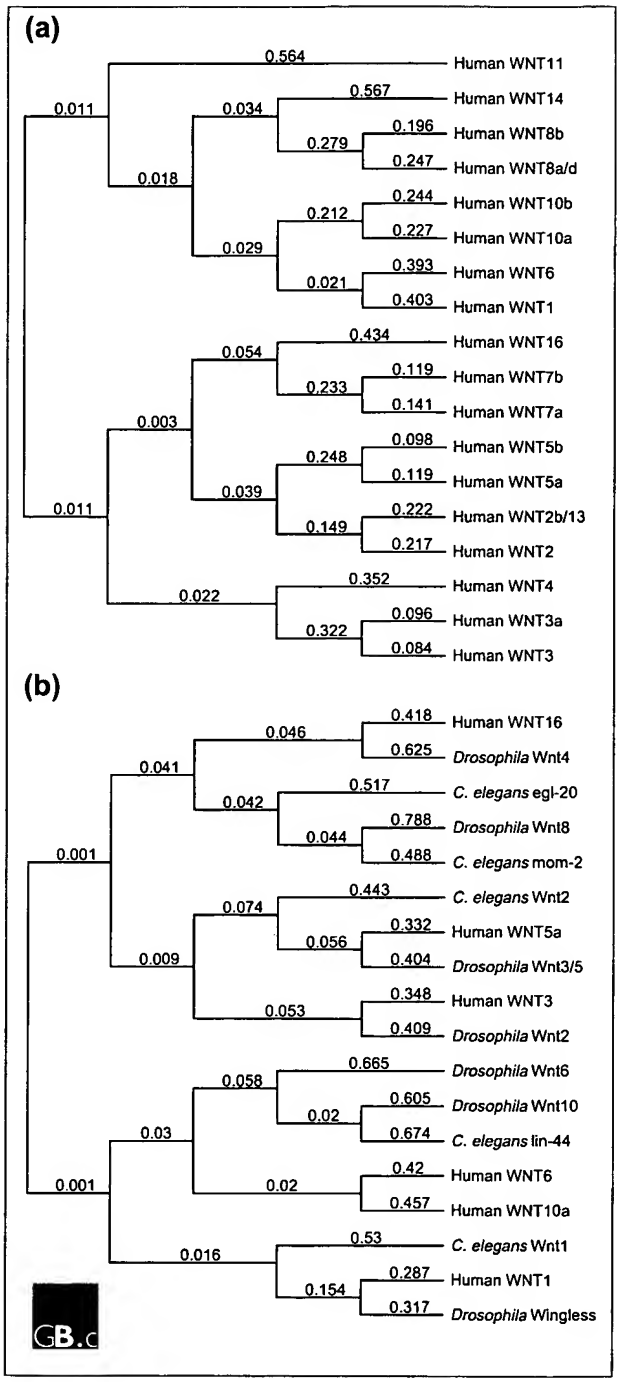

Figure 2
Predicted evolutionary relationships between members of the *Wnt* gene family. **(a)** Predicted relationships between 18 of the 19 known human *WNT* protein sequences; *WNT15* was omitted because only a partial sequence is available. **(b)** Predicted evolutionary relationships between selected human *WNT* proteins (representing each large grouping shown in (a)) and *Wnt* proteins from mouse, *Xenopus*, *Drosophila*, and *Caenorhabditis elegans*. Sequences were aligned using the ClustalW program; trees were constructed from the alignments using the neighbor-joining method and are diagrammed using midpoint rooting. Numbers indicate branch lengths.

proteoglycans play a role in sorting Wg to specialized membrane microdomains in Wg-producing cells or, alternatively, may play a role localizing Wg in distinct endocytic compartments in receiving cells.

Polarized distribution of *wg* transcripts in embryonic epithelial cells is also required for optimal signaling activity. High-resolution *in situ* hybridization analyses demonstrate that *wg* transcripts are localized apically in the embryonic epidermis and that this distribution is mediated by two *cis*-acting elements found in the 3′ UTR of the *wg* mRNA [29]. Mutation of these elements results in uniform localization of *wg* transcripts and impaired Wg protein distribution and signaling. The asymmetric distribution of *wg* transcripts is dependent on dynein-mediated microtubule transport [30].

Function

Wnts and Wnt receptors

Reception and transduction of Wnt signals involves binding of Wnt proteins to members of two distinct families of cell-surface receptors, members of the Frizzled (Fzd) gene family and members of the LDL-receptor-related protein (LRP) family [31,32]. The canonical Fzd receptor has an amino-terminal cysteine-rich domain (CRD) that binds Wnt, seven transmembrane domains and a short cytoplasmic tail containing a consensus PDZ domain binding motif (S/T-X-V in the single-letter amino-acid code) at the carboxyl terminus. The CRD forms a novel protein fold with a conserved dimerization interface that may be important for Wnt binding [33]. Fzd receptors have been identified in vertebrates and invertebrates; there are ten known members in humans and mice, four in flies, and three in worms. The general structure of Fzd receptors resembles that of seven-transmembrane G-protein-coupled receptors, suggesting that Fzd proteins may use heterotrimeric G proteins to transduce Wnt signals. Several recent studies provide evidence consistent with this idea, showing that a subgroup of Fzd receptors can signal through the pertussis-toxin-sensitive subclass of heterotrimeric G proteins to stimulate an increase in intracellular Ca^{2+} and activate protein kinase C (PKC) [34-38]. Heterotrimeric G proteins do not appear to be involved in transducing Wnt/Fzd signals that regulate the cytoskeleton-associated protein β-catenin, however (see below).

Two members of the vertebrate LRP family, LRP-5 and LRP-6, can bind Wnts and may form a ternary complex with a Wnt and a Fzd [39]. Mutations in *LRP-6* in mice result in developmental defects similar to those seen in mice deficient for several individual *Wnt* genes [40], and overexpression of LRP in *Xenopus* can activate the Wnt pathway [39]. In *Drosophila*, *arrow*, the ortholog of LRP5 and LRP6, is required for optimal Wg signaling [41]. Although the mechanism of LRP signaling is unclear, recent evidence suggests that binding of the cytoplasmic domain of LRP to the Wnt antagonist Axin may play a role in Wnt pathway activation [42].

In addition to the Fzd and LRP receptors, cell-surface proteoglycans also appear to have a role in the reception of Wnt signals. For example, genetic analyses in *Drosophila* have shown that several genes required for optimal Wg signaling encode cell-surface proteoglycans of the glypican family [43,44] and proteins involved in proteoglycan synthesis [45-47]. Furthermore, QSulf1, an avian protein related to heparan-specific *N*-acetyl glucosamine sulfatases, has also been shown to regulate heparan-dependent Wnt signaling in cultured cells [48]. It is unclear at this time how proteoglycans modulate Wnt signaling, but current suggestions include concentrating Wnt proteins at the cell surface or presenting Wnt ligands to cell-surface receptors.

Secreted modulators of Wnt signaling

Wnt signals are modulated extracellularly by diverse secreted proteins, including members of the Frizzled-related protein (FRP or FrzB) family [49], Wnt-inhibitory factor-1 (WIF-1) [50], Cerberus [51], and Dickkopf (Dkk) [52]. FRPs, WIF-1, and Cerberus can bind Wnt proteins directly and are thought to antagonize Wnt function by preventing their interaction with Fzd receptors. FRPs can also interact with Fzds, suggesting that a second way in which FRPs might antagonize Wnt signaling is through the formation of a nonfunctional complex with Fzd receptors. Humans have at least five *FRP* genes, and the specificity of each FRP for different Wnts remains to be determined. Dkk does not bind Wnts but instead interacts with the extracellular domain of LRPs, thereby blocking activation of Wnt signaling [42,53,54]. Four *Dkk* genes have been identified in vertebrates, including *Dkk2*, which does not act as a Wnt antagonist but rather can stimulate Wnt signaling [55].

Intracellular signaling pathways

Wnt signals are transduced through at least three distinct intracellular signaling pathways including the canonical 'Wnt/β-catenin' pathway, the 'Wnt/Ca^{2+}' pathway, and the 'Wnt/polarity' pathway (also called the 'planar polarity' pathway) [5,56-62]. Distinct sets of Wnt and Fzd ligand-receptor pairs can activate each of these pathways and lead to unique cellular responses. The Wnt/β-catenin pathway primarily regulates cell fate determination during development, whereas the major function of the Wnt/polarity pathway is regulation of cytoskeletal organization. The biological function of the Wnt/Ca^{2+} pathway is unclear.

The canonical Wnt/β-catenin pathway is intensely studied, and on the basis of current literature I propose the model illustrated in Figure 3a [59,63,64]. Signaling through this pathway depends on the levels of β-catenin in the cell. In the absence of Wnt, β-catenin is targeted for degradation by a multi-protein destruction complex. Wnt signaling antagonizes the destruction complex, leading to the accumulation of β-catenin and activation of target genes. Up-to-date lists of proteins involved in Wnt/β-catenin signaling and the potential roles of each of these proteins can be found on the worldwide web [5,60,62].

The Wnt/Ca^{2+} pathway involves an increase in intracellular Ca^{2+} and activation of PKC; it can be activated by a distinct group of Wnt ligands and Fzd receptors from those that activate other pathways, including Wnt5a, Wnt11 and Fzd2 (Figure 3b) [58,61,62]. The Wnt/Ca^{2+} pathway involves activation of a heterotrimeric G protein, an increase in intracellular Ca^{2+}, and activation of calcium/calmodulin-regulated kinase II (CamKII) and PKC [34,35,37]. The downstream targets of CamKII and PKC are currently unknown, but it has been shown that activation of the Wnt/Ca^{2+} pathway can antagonize the Wnt/β-catenin pathway in *Xenopus*, although it is unclear at what level this interaction occurs [65].

Wnt/polarity signaling regulates the polarity of cells through regulation of their cytoskeletal organization (Figure 3c) [56,57,62]. In vertebrates, Wnt/polarity signaling is thought to control polarized cell movements during gastrulation and neurulation [66-70]. In *Drosophila*, Wnt/polarity signaling

Figure 3 *(see the figure on the next page)*
The known Wnt signaling pathways. **(a)** In the Wnt/β-catenin pathway, Wnt signaling depends on the steady-state levels of the multi-functional protein β-catenin. In the absence of Wnt signal, a multi-protein destruction complex that includes the adenomatous polyposis coli protein (APC) and a member of the Axin family facilitates the phosphorylation of β-catenin by glycogen synthase kinase 3 (GSK3). GSK3 substrates also include APC and Axin; phosphorylation of each of these proteins leads to enhanced binding of β-catenin. Phosphorylated β-catenin is bound by the F-box protein β-TrCP, a component of an E3 ubiquitin ligase complex, and is ubiquitinated; the ubiquitin tag marks β-catenin for destruction by the proteasome. When a cell is exposed to a Wnt, the Wnt interacts with its coreceptors Frizzled and LRP. Activation of Frizzled and LRP leads to the phosphorylation of Dishevelled (Dsh), a cytoplasmic scaffold protein, perhaps through stimulation of casein kinase Iε (CKIε) and/or casein kinase II (CKII). Dsh then functions through its interaction with Axin to antagonize GSK3, preventing the phosphorylation and ubiquitination of β-catenin. In vertebrates, inhibition of GSK3 may involve the activity of GSK3 binding protein (GBP/Frat), which binds to both Dsh and GSK3 and can promote dissociation of GSK3 from the destruction complex. Unphosphorylated β-catenin escapes degradation, accumulates in the cell, and enters the nucleus, where it interacts with members of the TCF/LEF family of HMG-domain transcription factors to stimulate expression of target genes. In addition to the components of the Wnt/β-catenin pathway described here, many additional proteins with potential roles in regulating Wnt/β-catenin signaling have been reported including the phosphatase PP2A and the kinases Akt/protein kinase B, integrin-linked kinase (ILK), and PKC. **(b)** Signaling through the Wnt/Ca^{2+} pathway appears to involve activation of the two pertussis-toxin-sensitive G proteins, $G_{\alpha o}$ and $G_{\alpha t}$, in combination with $G_{\beta 2}$ [34,35]. G-protein activation then leads to an increase in intracellular Ca^{2+} and the subsequent stimulation of Ca^{2+}/calmodulin-dependent kinase II (CamKII) [37]. Activation of the Wnt/Ca^{2+} pathway also results in stimulation of PKC activity in the form of the translocation of PKC to the plasma membrane [34]. Downstream targets of the Wnt/$Ca2^{+}$ pathway have not been identified. **(c)** The Wnt/polarity pathway, which regulates cytoskeletal organization; the *Drosophila* Wnt/polarity pathway that regulates the polarity of trichomes in the wing is shown as an example. In this case, the nature of the polarity signal is not known.

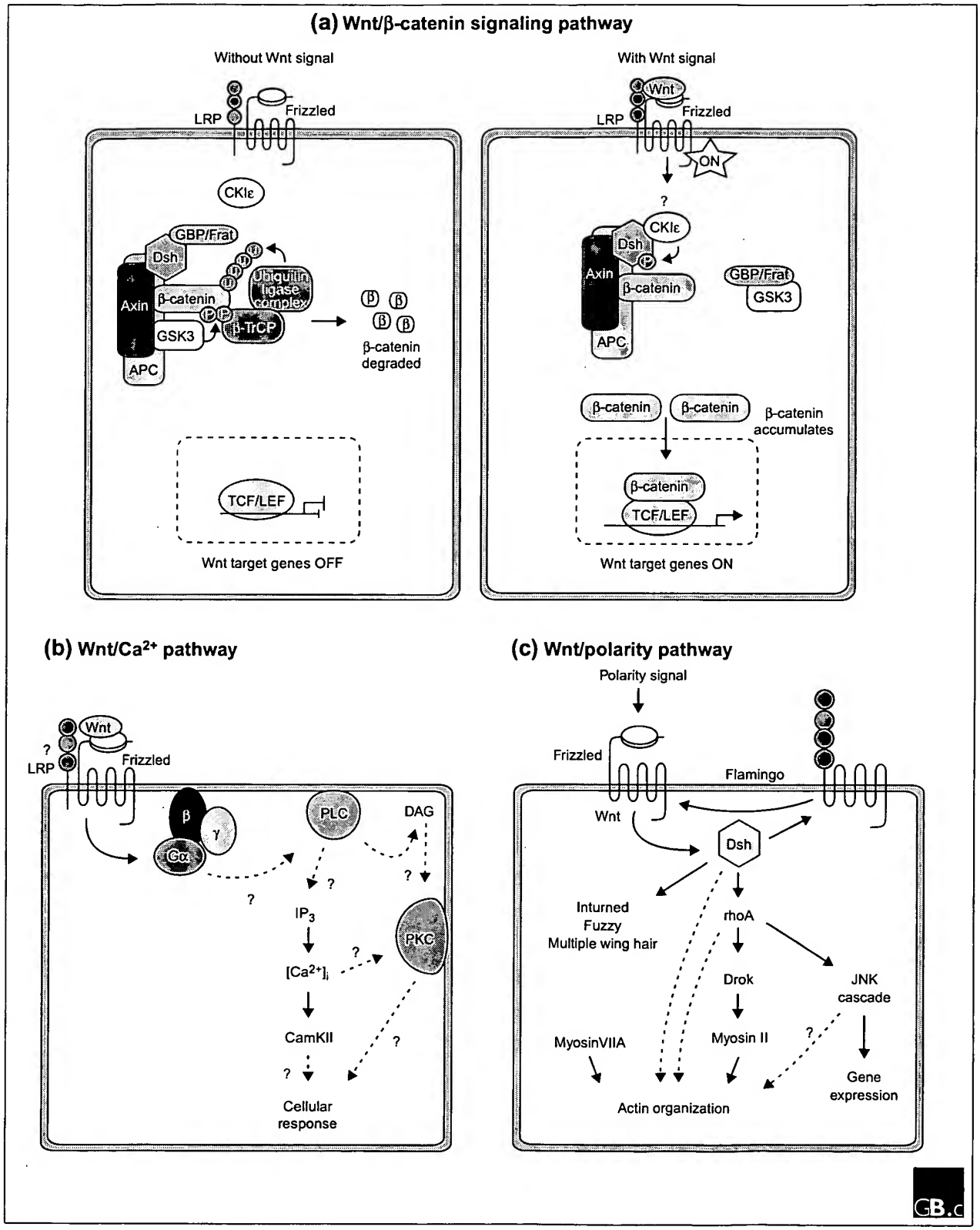

Figure 3 *(see the legend on the previous page)*

is required for the appropriate orientation of trichomes - or hairs - of the adult wing and for appropriate chirality of ommatidia in the eye, and may regulate asymmetric cell divisions of certain neuroblasts [56,71,72]. The only molecules known to function in both the vertebrate and the invertebrate Wnt/polarity pathways are members of the Fzd family and the cytoplasmic scaffold protein Dsh. The regulation of gastrulation movements in vertebrates also requires the activity of *Wnt11*, which may signal through Fzd7 to regulate protrusive activity during convergent extension [66,67]. In flies, genetic analyses have identified a number of potential components of the Wnt/polarity pathway in addition to DFzd1 and Dsh, including the small GTPase DrhoA, *Drosophila* rho-associated kinase (Drok), Jun N-terminal kinase (JNK), myosin II, myosin VIIA, and the products of the novel genes *flamingo/starry night*, *fuzzy*, *inturned*, and *strabismus/van gogh* [56,72]. A Wnt ligand for the Wnt/polarity pathway has not been identified in flies, however, and it remains to be seen how much of the intracellular signaling mechanism has been conserved between vertebrates and invertebrates.

Several studies have suggested that distinct classes of Wnts signal through either the Wnt/β-catenin pathway or the Wnt/Ca^{2+} pathway [58]; for example, overexpression studies in *Xenopus* have shown that XWnt1, XWnt3a, XWnt8, and XWnt8b can stimulate the Wnt/β-catenin pathway whereas XWnt4, XWnt5a, and XWnt11 can stimulate the Wnt/Ca^{2+} pathway [58]. Furthermore, the separation of Wnts into these two distinct functional classes is mirrored by the classification of Fzd proteins into similar functional groups on the basis of their ability to activate one or other pathway in overexpression assays. Although this classification of Wnts, which partially mirrors their evolutionary relationships, may provide a useful tool for predicting the function of Wnts and Fzds, the relationship between specific Wnts and the intracellular pathway they use is not fixed. For example, overexpression of XWnt5a in combination with human FZD5 in *Xenopus* embryos results in activation of the Wnt/β-catenin pathway [73], suggesting that the activity of Wnts *in vivo* will be determined by the repertoire of Fzd receptors present at the cell surface.

Important mutants and developmental functions

Loss-of-function mutations in 9 of the 18 mouse *Wnt* genes have been generated, and the phenotypes of mutant embryos demonstrate the diverse functions of *Wnt* genes during embryogenesis (Table 2). For example, knocking out *Wnt1* results in a dramatic loss of a portion of the midbrain and deletion of the rostral cerebellum [74,75]. Inactivation of *Wnt4* results in the absence of kidneys [76], masculinization of mutant females (absence of the Müllerian duct and continued development of the Wolffian duct) [77], and defects in mammary gland morphogenesis during pregnancy [78]. Targeted knockout of *Wnt7a* also has pleiotropic effects, including ventralization of the limbs

Table 2

Developmental functions of mouse *Wnt* genes

Gene	Natural allele	Phenotype of knockout or other functions	References
Wnt1	*swaying*	Loss of a portion of the midbrain and cerebellum	[74,75,129,130]
		Deficiency in dorsal neural-tube derivatives, including neural-crest cells in double knockout with *Wnt3a*	[131]
Wnt2		Placental defects	[132]
Wnt3		Defects in axis formation and gastrulation	[84]
		Defects in hair growth and structure	[133,134]
Wnt3a	*vestigial tail*	Defects in somite and tailbud development	[102,135-137]
		Deficiency in dorsal neural-tube derivatives, including neural crest cells in double knockout with *Wnt1*	[131]
		Loss of hippocampus	[138]
Wnt4		Defects in kidney development	[76]
		Defects in female development; absence of Müllerian duct, ectopic synthesis of testosterone in females	[77]
		Defects in mammary gland morphogenesis	[78]
Wnt5a		Truncated limbs, shortened anterior-posterior axis, reduced number of proliferating cells	[139]
Wnt7a	*postaxial hemimelia*	Defects in limb polarity	[79]
		Female infertility due to failure of Müllerian duct regression	[80,140]
		Defects in uterine patterning	[141]
		Defects in synapse maturation in the cerebellum	[81]
Wnt7b		Placental defects	[142]
Wnt10b		Inhibition of adipogenesis	[143]

[79], female infertility due to failure of Müllerian-duct regression [80], and a delay in the morphological maturation of glomerular rosettes in the cerebellum [81].

Overexpression and antisense 'knockdown' analyses in *Xenopus* have shown that the Wnt/β-catenin pathway is required for the specification of dorsal cell fates [82]. A debate is ongoing, however, over whether a maternal Wnt ligand is required to activate this pathway in dorsal cells. In support of a role for a Wnt ligand, a recent study has shown that *XFzd7* is important for establishing dorsal cell fates [83], thereby implicating a Wnt ligand in this process. Furthermore, targeted knockout of *Wnt3* in mice results in defects in axis formation and gastrulation, suggesting a conserved role for Wnts in regulating the establishment of the dorsal-ventral axis in vertebrates [84]. On the other hand, overexpression of a dominant-negative form of *Xwnt8* in oocytes does not suppress formation of dorsal cell fates, arguing against the requirement for a maternal Wnt in axis specification [10]. Further studies are necessary to resolve the role of Wnts in vertebrate early axial development.

In flies, Wnt signaling has a variety of functions during development. The *wg* gene is required for cell-fate choices in the ventral epidermis during embryogenesis, as well as for many other functions, and *DWnt2* is required for testis and adult muscle development [17]. In *C. elegans*, genetic analyses have defined a number of roles for Wnts, including establishment of polarity and endodermal cell fates in the early embryo and regulation of cell migration, among many others [85]. A comprehensive list of *Wnt* genes and their mutant phenotypes in vertebrates and invertebrates can be found at the *Wnt* gene homepage [5].

Wnt signaling and cancer

In addition to the many roles for Wnt signaling during development and in adult tissues, it is also involved in tumorigenesis in humans [59,64]. Although mutation or misexpression of a *Wnt* gene has not been linked directly to cancer in humans, mutation of several intracellular components of the Wnt/β-catenin pathway is thought to be critical in many forms of cancer. Most notably, patients with familial adenomatous polyposis (FAP) develop multiple intestinal adenomas early in life and have germline mutations in the *APC* gene. In addition, mutation of *APC* is associated with more than 80% of sporadic colorectal adenomas and carcinomas. More than 95% of germline and somatic mutations of the *APC* gene are nonsense mutations that result in the synthesis of a truncated protein lacking the region of APC that is important for its function in the destruction complex. Significantly, these truncations in APC remove binding sites for β-catenin and Axin, as well as putative phosphorylation sites for GSK3; as a result, the mutant APC protein cannot efficiently promote degradation of β-catenin. Mutations in the third exon of the human β-catenin gene (*CTNNb1*) that make it refractory to phosphorylation-dependent degradation and lead to inappropriate accumulation of β-catenin have also been identified in a large number of primary human cancers (see [64] for a table of β-catenin mutations in human cancers). Interestingly, mutations in *CTNNb1* and *APC* are rarely found in the same tumor; for example, in colon cancer, in which the vast majority of tumors have mutations in *APC*, the overall frequency of *CTNNb1* mutations is relatively low, but colorectal tumors lacking *APC* mutations are much more likely to have mutations in *CTNNb1*. Recently, Axin has also been shown to act as a tumor suppressor; mutations in the *Axin1* gene have been found in human hepatocellular cancers [86]. Importantly, mutations in *Axin1* and *CTNNb1* found in hepatocellular carcinomas also show mutual exclusivity similar to that seen for *APC* and *CTNNb1* in colon cancers. Together, these data strongly argue that mutations resulting in the stabilization of β-catenin can promote cancer in many tissue types.

Frontiers

The large number of *Wnt* genes and the many roles that Wnt signaling plays in development and human disease pose many unresolved issues for researchers. One of the major unanswered questions is the specificity of interactions between different Wnt ligands and Fzd receptors and also which downstream pathways these many different ligand-receptor pairs stimulate. It also remains unclear how Wnt signals are transduced by the Fzd-LRP receptor complex and what role proteoglycans play in this process. Inside the cell, many questions regarding the transduction of Wnt signals remain, including how receptor activation stimulates Dsh and how Dsh discriminates between different Wnt signals to activate either the Wnt/β-catenin or the Wnt/polarity pathway. Furthermore, many roles of *Wnts* during development remain to be determined. This challenge will require detailed analyses of knockout mice, in addition to biochemical, cell-biological and genetic analyses in other model systems, to characterize the functions of Wnts and the signaling pathways they use during embryogenesis. Finally, the identification and characterization of mutations in Wnt-pathway genes involved in human disease is ongoing and these studies, together with a greater knowledge of the molecular mechanism of Wnt signal transduction, promise future clinical therapies for devastating human afflictions such as colon cancer. Thus, although there is so much still to learn, the importance and widespread occurrence of Wnt signaling guarantees the rapid increase in our understanding of the normal and abnormal functions of the Wnts.

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Correspondence and requests for materials should be addressed to K.N. (knomuscb@mbox.nc.kyushu-u.ac.jp) or K.S. (k-sugar@kobepharma-u.ac.jp). Sequences of the longer ChSy and the shorter ChSy have been deposited in the DNA Data Bank of Japan under accession numbers AB088397 and AB088398, respectively.

Wnt proteins are lipid-modified and can act as stem cell growth factors

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Wnt signalling is involved in numerous events in animal development[1], including the proliferation of stem cells[2] and the specification of the neural crest[3]. Wnt proteins are potentially important reagents in expanding specific cell types, but in contrast to other developmental signalling molecules such as hedgehog proteins and the bone morphogenetic proteins, Wnt proteins have never been isolated in an active form. Although Wnt proteins are secreted from cells[4–7], secretion is usually inefficient[8] and previous attempts to characterize Wnt proteins have been hampered by their high degree of insolubility. Here we have isolated active Wnt molecules, including the product of the mouse *Wnt3a* gene. By mass spectrometry, we found the proteins to be palmitoylated on a conserved cysteine. Enzymatic removal of the palmitate or site-directed and natural mutations of the modified cysteine result in loss of activity, and indicate that the lipid is important for signalling. The purified Wnt3a protein induces self-renewal of haematopoietic stem cells, signifying its potential use in tissue engineering.

We expressed several Wnt genes, including *Wnt3a* (ref. 9), in a variety of cell lines and generated antibodies to monitor Wnt protein secretion into the medium. For purification purposes, we selected clones of cells secreting the highest amounts of protein (200 ng ml^{-1} for Wnt3a from mouse L (L-M[TK$^-$]) cells). We tested the activity of Wnt3a by assaying its ability to stabilize cytosolic β-catenin, a known target and signal transduction component of Wnt signalling[10]. Mouse L cells accumulate high levels of β-catenin protein after a 2-h incubation with Wnt3a-conditioned medium (Fig. 1b, top panel; see also ref. 11).

Initial characterization of secreted Wnt3a indicated that it is hydrophobic (see below), therefore we designed a purification protocol that starts with chromatography on blue (Cibacron blue 3GA) Sepharose in the presence of the detergent CHAPS. Under these conditions, Wnt3a binds with high selectivity to the resin and can be eluted in a relatively pure form by increasing ionic strength (Fig. 1a and Table 1). Approximately 60% of added Wnt3a is recovered in this step with nearly 2,500-fold enrichment. We then separated Wnt-containing fractions by size exclusion chromatography on a Superdex 200 column, and finally by cation exchange on heparin (Table 1). These steps yielded fractions of Wnt3a that were greater than 95% pure as assessed by Coomassie staining (Fig. 1a). Through size exclusion chromatography, we determined that active Wnt3a is monomeric (not shown).

We have applied successfully similar purification methods to a variety of other Wnt proteins, including *Drosophila* Wnt8 (Fig. 1a), mouse Wnt5a and *Drosophila* Wingless (not shown).

Throughout the purification, we measured the ability of Wnt3a to stabilize β-catenin in L cells. The final purified product exhibited no loss in activity compared to the original starting material (Fig. 1b). The purified Wnt3a protein retains the range of activities expected for a Wnt protein. For example, we tested the effect of Wnt3a protein on *Xenopus* animal cap explants and found that two known target genes, *siamois* and *Xnr3* (refs 12, 13), are induced by Wnt3a (Fig. 1c). As a further assay for Wnt activity, we used C57MG cells, a line derived from the mouse mammary gland that can be morphologically transformed by *Wnt* gene expression[8]. Purified Wnt3a promotes the morphological transformation of these cells (Fig. 1d) similar to that of *Wnt* gene transfection. Furthermore, the protein can induce expression of known transcriptional Wnt targets including *MSX1*, cyclin D1 and *MYC* in human teratocarcinoma cells (data not shown).

All purification steps required the presence of detergent to maintain solubility and activity, suggesting that Wnt proteins are hydrophobic. We used the two-phase separation property of the detergent Triton X-114 (ref. 14) to test this. Most Wnt3a partitioned to the detergent phase (Fig. 2a), a behaviour characteristic of highly hydrophobic proteins such as integral membrane proteins. As the primary amino acid sequence of secreted Wnt does not contain long stretches of hydrophobic residues, we used metabolic labelling to test whether Wnt is post-translationally modified by lipid attachment. We found that the protein is labelled with tritiated palmitate (Fig. 2b).

Evidence for the functional importance of the lipid modification

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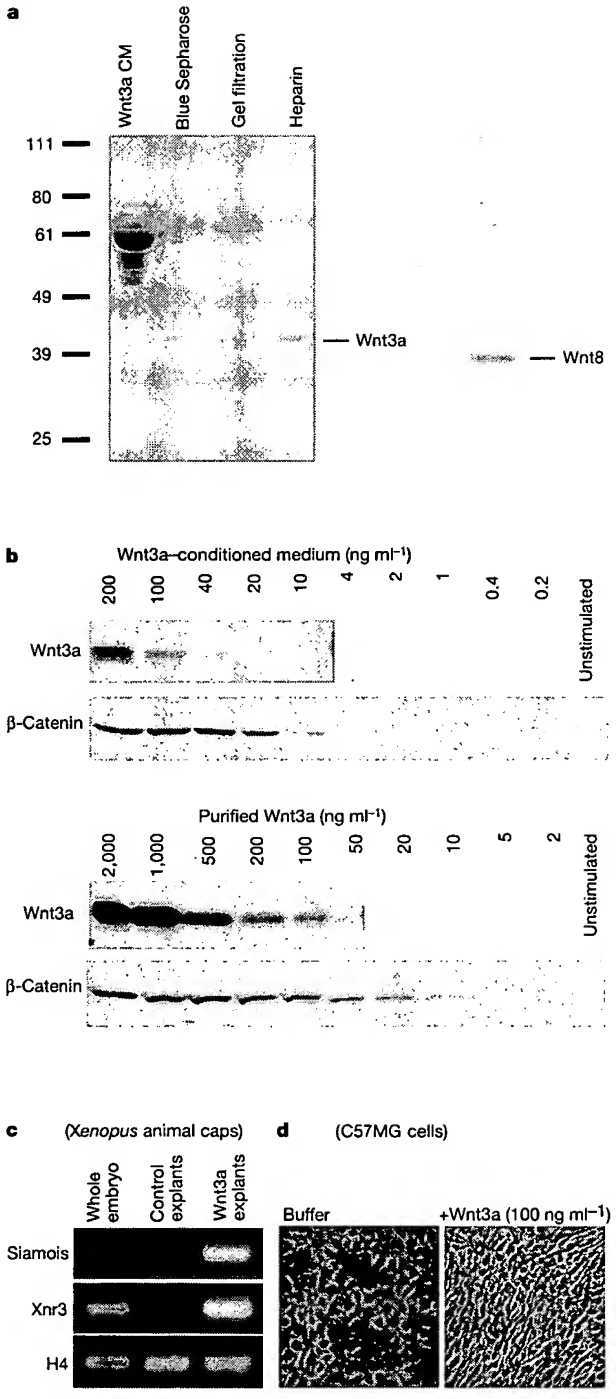

Figure 1 Wnt3a and *Drosophila* Wnt8 purification. **a**, Coomassie staining of an SDS polyacrylamide gel containing fractions from all steps of the purification reveals the enrichment of the Wnt3a protein. Also shown is the final *Drosophila* Wnt8 fraction, purified using the same protocol. Size markers are in kilodaltons. **b**, Wnt3a stabilizes the β-catenin protein. Wnt3a-conditioned medium (200 ng ml^{-1}) and purified Wnt3a (100 μg ml^{-1}) was diluted as indicated in medium containing 10% FBS and detected by western blot. L cells were stimulated for 2 h. **c**, Wnt3a induces expression of *siamois* and *Xnr3* in animal cap explants of *Xenopus* embryos. Animal cap explants were incubated with 100 ng ml^{-1} Wnt3a and analysed by polymerase chain reaction with reverse transcription for expression of the direct targets *Xnr3* and *siamois*. **d**, Wnt3a induces the morphological transformation of C57MG cells. C57MG cells were treated with or without 100 ng ml^{-1} Wnt3a for 2 days in serum-containing medium and then an additional 2 days in serum-free medium.

came from treatment of Wnt3a with acyl-protein thioesterase-1 (APT-1), an enzyme that removes palmitate from G proteins and other thioacyl protein substrates[15]. This treatment shifts Wnt3a to the water phase in the Triton X-114 phase separation experiment (Fig. 2c), suggesting that APT-1 removes a thioester-linked lipid, such as palmitate. APT-1 also blocks the ability of Wnt3a to stabilize β-catenin (Fig. 2c).

To map the lipid attachment site on the Wnt polypeptide we subjected proteolytic peptide fragments of both Wnt3a and *Drosophila* Wnt8 to liquid chromatography tandem mass spectrometry, which identifies molecular masses of the ionized peptides and obtains primary amino acid sequence information through collision-induced fragmentation. In both proteins we identified ions whose masses were consistent with the addition of 238 daltons (the mass of palmitate is 256 daltons accounting for the loss of water in the formation of a thioester linkage) and which produced fragmentation data consistent with a peptide containing a conserved cysteine modified by palmitate (C77 in Wnt3a and C51 in *Drosophila* Wnt8; underlined in Fig. 2d). This cysteine is absolutely conserved among all Wnt family members (bold in Fig. 2d); it is the most amino-terminally conserved cysteine of the Wnt family (http://www.stanford.edu/~rnusse/genealigns/manywnts.html).

To test for the requirement of C77 in cell culture, we mutated it to alanine in Wnt3a and expressed the mutant protein (Wnt3a(C77A), Fig. 2e) in 293 and in L cells. The mutant Wnt3a protein was secreted at levels similar to that of the wild-type protein. This indicated that the mutation, unlike many other cysteine mutations in Wnt proteins[16], does not interfere with the folding of the protein. However, when the Wnt3a(C77A) protein was subjected to the Triton X-114 phase separation test, it partitioned in the water phase, indicating that it had lost its hydrophobic character (Fig. 2a). In a β-catenin assay on L cells, Wnt3a(C77A) was not active over a range of concentrations tested (Fig. 2e, left). In a transfection assay on 293 cells however, there was a noticeable increase in the intracellular levels of β-catenin, demonstrating that the Wnt3a(C77A) mutant retains some activity when expressed at high levels in an autocrine manner (Fig. 2e, right).

Notably, a natural loss-of-function allele of the *Caernorhabditis elegans egl-20* gene (*egl-20(N585)*; ref. 17) contains a serine replacing the cysteine corresponding to C77 (Fig. 2d). Moreover, in a survey of *wingless* (*wg*) alleles in *Drosophila*, we found that the wg^{S21} allele[18] contains a tyrosine instead of that same cysteine (Fig. 2d). Thus, our data are consistent with the lipid modification being important for Wnt signalling activity. At the moment, we cannot exclude the possibility that Wnt proteins carry other modifications beyond palmitoylation and *N*-linked glycosylation; nor can we rule out that different forms of Wnt proteins (that is, cell-bound) are palmitoylated at other sites.

Next we investigated whether Wnt3a can be used as a reagent to control cell fate in a well-characterized stem cell system, through application of the isolated protein to purified haematopoietic stem cells (HSCs)[19]. Single HSCs responded well to the Wnt3a protein in the presence of limiting doses of steel factor (SLF). Over a period of 7 days, the frequency of cells proliferating was 5.8-fold greater compared with control conditions (Fig. 3a, b). Most of the cells (82%) were undifferentiated, as they did not express markers for differentiated lineages. Thirty per cent of the lineage-negative cells expressed c-Kit and Sca-1, consistent with an HSC phenotype, whereas 64% were at the stage of myeloid progenitors (c-Kit$^+$, Sca-1$^-$; Fig. 3c, d). In contrast, incubation of HSCs with unfractionated Wnt3a-conditioned medium, in which Wnt3a itself is present at a similar concentration, resulted in a significant fraction (86%) of the cells expressing markers specific for differentiated lineages (Fig. 3c). This suggests that conditioned medium contains factors not present in purified Wnt3a that promote differentiation, underscoring the importance of having purified Wnt proteins available for the purpose of maintaining the self-renewing fate of HSCs.

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Table 1 **Purification table**

	Volume (ml)	Protein concentration	Total protein (mg)	Wnt3a concentration	Wnt3a (μg)
Wnt3a CM	2,000	4.46*	8,920	200‡	400
Blue Sepharose	60	36.0†	2.16	4†	240
Gel filtration	36	17.1†	0.615	5†	180
Heparin cation exchange	1.15	104†	0.120	100†	115

The concentration of Wnt3a protein in the conditioned medium (CM) was determined by comparing its signal intensity on a Wnt3a immunoblot to that of a serial dilution of a known amount of purified Wnt3a protein.
*Concentration in mg ml^{-1}.
†Concentration in μg ml^{-1}.
‡Concentration in ng ml^{-1}.

To determine whether the cells that proliferated in response to Wnt3a truly maintained HSC activity, we carried out a transplantation analysis. Single HSCs were plated in Terasaki plates and treated with Wnt3a or control medium for a period of 6 days. In previous experiments we showed that culturing cells with SLF alone (our control conditions) while inducing proliferation does not induce self-renewal *in vitro*[19]. Each well containing cells that responded to Wnt3a from a single cell was separately injected into lethally irradiated mice, and analysed after 6 weeks of reconstitution (Fig. 3e). If no self-renewal had occurred, only 10% of the mice would be expected to be reconstituted successfully (see Fig. 3 legend). In contrast 100% of the transplanted mice contained donor-derived cells (Fig. 3f), suggesting that HSCs had undergone self-renewal in response to purified Wnt3a.

We have established methods to purify significant quantities of pure and active Wnt proteins, which can be used for self-renewal of HSCs and potentially other stem cells. We found that Wnt proteins are unexpectedly hydrophobic and are post-translationally modified by palmitoylation, a property that explains the poor solubility of the proteins. It is interesting to note that the protein products of the *Drosophila porcupine* and *C. elegans mom-1* genes[20,21] have homology with acyl transferases and may catalyse Wnt acylation[22]. Moreover, the Porcupine protein can bind to a domain in Wingless encompassing the acylation site[23]. *porcupine* and *mom-1* have phenotypes similar to *Wnt* alleles and are required in Wnt-producing cells, indicating that the lipid is an integral part of signalling activity. However, overexpression of Wingless in the *Drosophila* embryo can overcome the absence of *porcupine*[24], just as high expression of Wnt3a(C77A) can lead to a modest increase in β-catenin (Fig. 2d). This suggests that the lipid functions to increase the local concentration of Wnt on membranes, and that its absence can be overcome by high levels of expression. Although palmitoylation of secreted proteins seems unusual, there is an intriguing

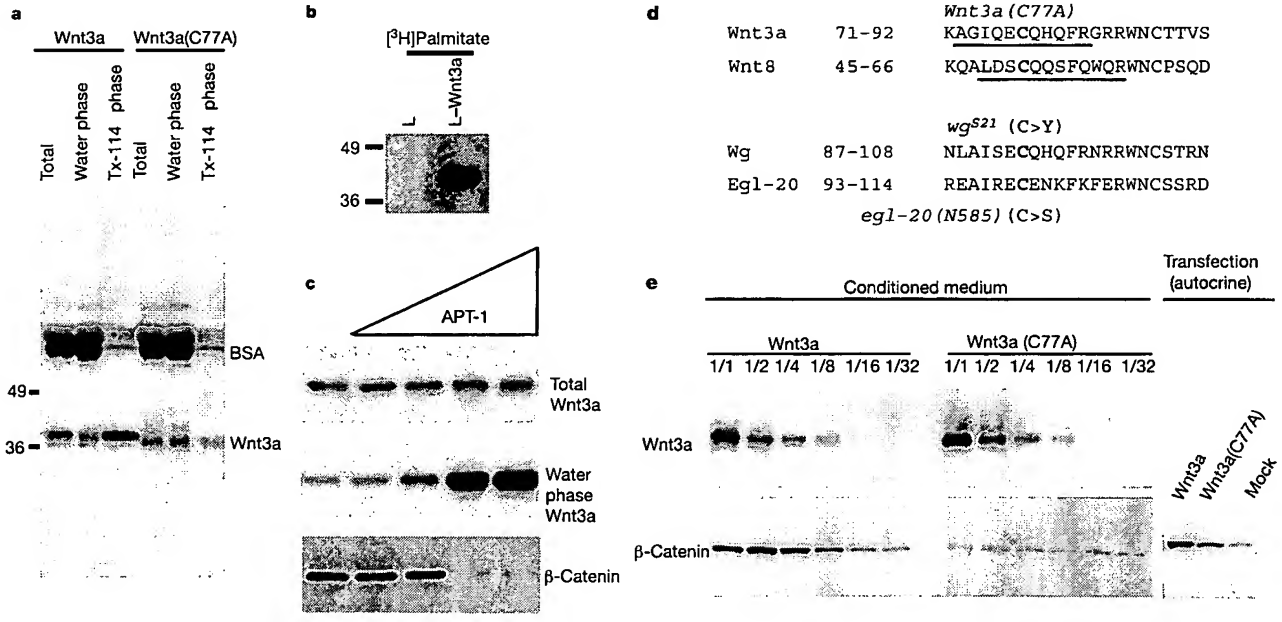

Figure 2 Wnt proteins are palmitoylated on an essential cysteine. **a**, Triton X-114 phase separation (western blot). Most wild-type Wnt3a separates to the Triton X-114 phase, indicating that it is hydrophobic, but the Wnt3a(C77A) mutant (see **d**) partitions mostly to the water phase. BSA from serum partitions to the water phase and serves as an internal control. **b**, *In vivo* labelling of Wnt3a protein with tritiated palmitate. Wnt3a was partially purified from conditioned medium of cells labelled with tritiated palmitate for 5 h. **c**, APT-1 treatment of Wnt3a (western blot). Treatment of Wnt3a with increasing amounts of APT-1 shifts the Wnt3a protein from the Triton X-114 phase (data not shown) to the water phase (middle panel) and abolishes its activity in the β-catenin stabilization assay. **d**, Mass spectrometry maps the palmitate modification to a cysteine (bold) in Wnt3a (C77) and in *Drosophila* Wnt8 (C51). Underlined sequence corresponds to the peptide identified in the spectra as being modified. The cysteine is conserved in all known Wnt proteins. A site-directed mutant (Wnt3a(C77A)) was made and used in **a** and **e**. The *Drosophila* wg^{S21} (ref. 18) allele has a mutation converting the cysteine into a tyrosine and the *egl-20(N585)* allele in *C. elegans* has a serine instead of the cysteine[17]. These are both loss-of-function alleles. **e**, The Wnt3a(C77A) mutant protein is secreted from 293 cells at levels similar to wild type, but is not active in increasing β-catenin in target L cells over a range of concentrations tested (western blot). However, the 293 cells transfected with the Wnt3a(C77A) expression construct show a modest increase in β-catenin, indicating that high levels of the mutant can activate Wnt signalling. The Wnt3a(C77A) and wild-type transfected cells express equal levels of Wnt protein.

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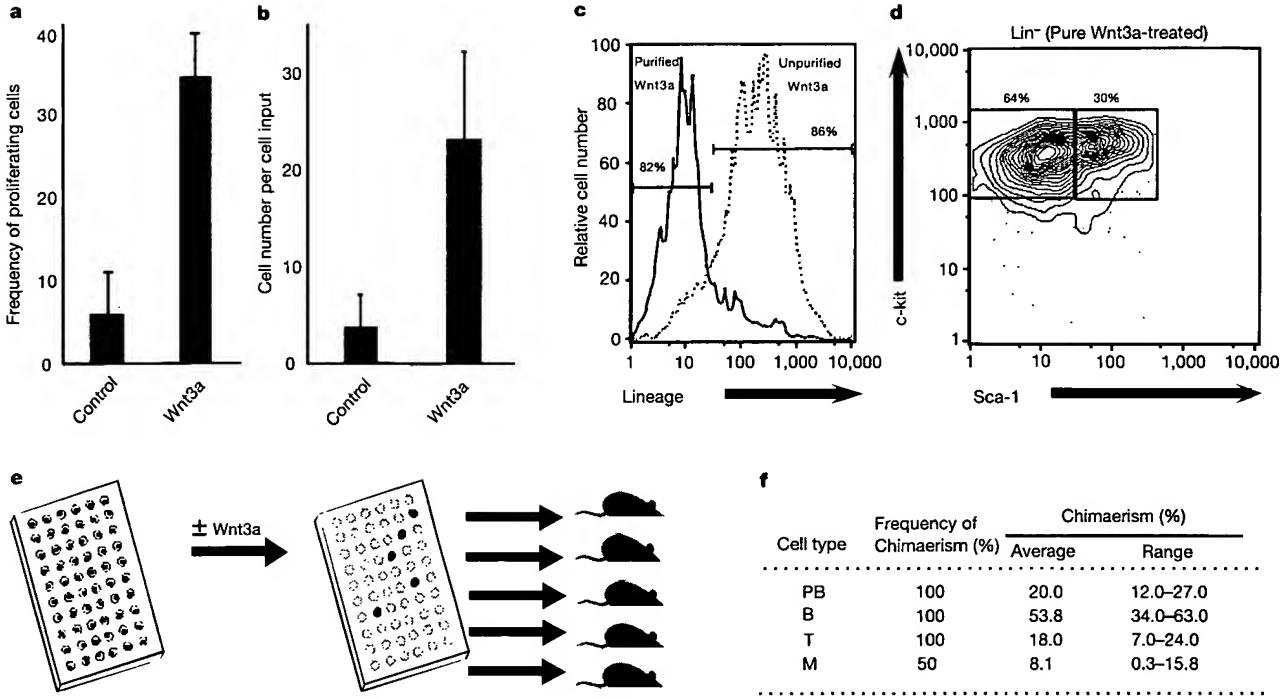

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Cell type	Frequency of Chimaerism (%)	Chimaerism (%) Average	Chimaerism (%) Range
PB	100	20.0	12.0–27.0
B	100	53.8	34.0–63.0
T	100	18.0	7.0–24.0
M	50	8.1	0.3–15.8

Figure 3 HSCs maintain self-renewing fate with reduced differentiation in response to purified Wnt3a. Purified mouse bone marrow (BM) HSCs (c-Kit$^+$, Sca-1$^+$, Thy-1.1^{lo}, Lin$^-$) from Bcl2 transgenic mice[19] were sorted by FACS and plated as single cells into 60-well Terasaki plates. Cells were incubated in X-vivo15 (Bio Whittaker) containing either purified Wnt3a (about 100 ng ml^{-1}) plus limiting amounts of SLF (7.5 ng ml^{-1}) or SLF (7.5 ng ml^{-1}) alone, as a control. **a**, Cell growth was monitored over 7 days in culture, and shown as the frequency of responding cells. **b**, Total cell growth. Cells responded to Wnt3a by proliferating >100-fold (from 1 cell to at least 100 cells) and the total number of cells generated was sixfold greater in the presence of Wnt3a than control conditions. Results are representative of four independent experiments. **c**, To determine phenotypic characteristics, cells were plated in 96-well plates and incubated in the presence of purified or unpurified Wnt3a. After 7 days in culture, most cells treated with purified Wnt3a (at 100 ng ml^{-1}) were negative for lineage markers (solid line) whereas most treated with unpurified Wnt3a (200 ng ml^{-1} in the medium; Table 1) strongly upregulated lineage markers (dotted line). **d**, FACS analysis of purified Wnt3a-treated cells. The lineage-negative population is distributed into c-Kit$^+$ and Sca-1$^+$ HSCs and c-Kit$^+$ and Sca-1$^-$ myeloid progenitors. **e**, Purified mouse BM HSCs were plated singly into 60-well Terasaki plates and treated with Wnt3a for 6 days, then all cells generated from the single cell were transplanted individually into lethally irradiated mice along with 300,000 rescuing BM cells. **f**, Peripheral blood (PB) from each transplanted mouse was analysed after 6 weeks for reconstitution along both lymphoid (B and T) and myeloid (M) lineages. On the basis of the reconstitution efficiency of single transplanted HSCs, 1 of 10 (10%) resting HSCs and probably 1 of 50 (2%) cycling HSCs reconstitute. A 50% reconstitution rate suggests at least a fivefold and probably a 15–25-fold expansion in HSCs per transplant. Fivefold expansion is probably an underestimate as HSCs transplanted in low numbers lead to low and variable reconstitution. But our finding that Wnt3a-treated HSCs on transplantation lead to an average chimaerism of 20% (range 12–27%) in the context of a competitive reconstitution suggests a greater than fivefold expansion of functional HSCs.

parallel between Wnt and hedgehog signalling, as the hedgehog protein is also palmitoylated[25]. □

Methods

Purification of Wnt3a

Mouse L cells (American Type Culture Collection (ATCC) CRL-2648) were cultured in DMEM medium, 10% fetal bovine serum (FBS) and antibiotics. These cells were stably transfected with a vector containing the Wnt3a complementary DNA under the control of the PGK promoter, and G418-resistant clones were selected and screened for production of Wnt3a protein (ATCC CRL-2647). *Drosophila* S2 cells were used to produce the *Drosophila* Wnt8 protein, which was expressed from a heat-shock promoter[26]. Two litres of 0.2-μm filtered medium from L-Wnt3a cells, conditioned for 4 days, was adjusted to 1% Triton X-100, filtered and applied to blue (Cibacron blue) Sepharose HP (Amersham Biosciences) column (bed volume of 120 ml), which was previously equilibrated in binding buffer (150 mM KCl, 20 mM Tris-HCl, 1% CHAPS, pH 7.5). The column was then washed with four column volumes of binding buffer. Bound proteins were eluted with a single step to 1.5 M KCl, 20 mM Tris-HCl, 1% CHAPS, pH 7.5. Wnt3a eluted in two pools, each of which contained similar amounts of Wnt3a protein; however, the second pool contained significantly less total protein than the first (30.6 mg total protein in the first pool and 2.16 mg in the second pool). Fractions from this second pool were combined, concentrated to 12.5 ml on a Centricon 30 ultrafiltration device (Amicon), and fractionated on a HiLoad 26/60 Superdex 200 column (Amersham Biosciences) in phosphate buffered saline (PBS), 1% CHAPS, pH 7.3. Fractions containing Wnt3a were then fractionated on a 1-ml HiTrap Heparin column (Amersham Biosciences) in a single step elution from PBS, 1% CHAPS to PBS, 1% CHAPS, 1 M NaCl. N-terminal sequence of 1 μg purified Wnt3a was obtained by automated Edman degradation on a Procise 494 ABI sequenator. Isolated Wnt3a begins with residue 19 of the predicted amino acid sequence (SYPIWWSLAVGPQYS), indicating that the protein is proteolytically processed to remove the signal sequence. For a detailed protocol, see http://www.stanford.edu/~rnusse/wntwindow.html.

Triton X-114 phase separation

Wnt3a-conditioned medium was mixed 1:1 with ice cold 4.5% Triton X-114, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, incubated on ice for 5 min, then at 31 °C for 5 min, and centrifuged at 2,000*g* at 31 °C for 5 min. The top, aqueous phase was separated from the bottom Triton X-114 phase and equal volumes were immunoblotted with the anti-Wnt3a antibody.

In vivo labelling of Wnt3a with palmitate

L and L-Wnt3a cells were cultured in 10-cm plates for 3 days after a 1:10 split. [9,10(n)-3H] palmitic acid (Amersham Biosciences) was added to the medium at a concentration of 0.1 mCi ml^{-1} and incubated for 5 h at 37 °C. The medium was filtered, CHAPS was added to a concentration of 1%, and then re-filtered. The individual medium was fractionated on 1-ml HiTrap blue Sepharose columns (Amersham Biosciences) as described above. The Wnt3a-containing fractions or analogous fractions were precipitated with trichloroacetic acid and analysed by gel-electrophoresis and autoradiography.

Liquid chromatography tandem mass spectrometry

Purified Wnt3a and *Drosophila* Wnt8 were precipitated with trichloroacetic acid, re-suspended, alkylated and reduced as described[27]. The sample was split into three aliquots, digested separately with trypsin, subtilisin and elastase, and the resulting peptide mixtures

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were recombined and analysed by MudPIT as described[28] with modifications as described[27] on a Finnigan LCQ-Deca. Tandem mass spectra were searched against a database of predicted open reading frames to which common contaminants such as keratin and trypsin were added. Search results were filtered and grouped using the DTASelect program[29] and identifications confirmed through manual evaluation of spectra. The data were subsequently searched with a differential modification on cysteine of 238 to identify sites of palmitoylation. We also observed this peptide in its unpalmitoylated form, and at present we cannot distinguish whether the lipid is labile and lost during the manipulation of the sample or whether there is a pool of unmodified Wnt3a present in the preparation. We found the following masses [(M + H) +]: Wnt3a peptide unmodified: 1374.51 (predicted, 1374.465); Wnt3a peptide modified: 1556.10 (predicted, 1555.465); *Drosophila* Wnt8 peptide unmodified: 1583.37 (predicted, 1583.667); *Drosophila* Wnt8 peptide modified: 1764.23 (predicted, 1764.667). Although the tandem mass spectrometry analysis of Wnt3a and *Drosophila* Wnt8 identified 85% and 90% of the primary amino acid sequences, respectively, we did not obtain evidence for additional lipid modifications on other residues (S, T, Y, K, R).

Acyl-protein thioesterase treatment of Wnt3a

A total of 100 ng Wnt3a was treated in the presence of 1 μg BSA with 1, 10, 100 or 1,000 ng APT-1 (provided by A. Gilman) in buffer (PBS, 1% CHAPS, 1 M NaCl) in a total volume of 10 μl and incubated overnight at 30 °C. The reaction products were analysed in the β-catenin stabilization assay on L cells and in the Triton X-114 phase separation assay.

HSC isolation and assays

HSCs were sorted from mouse bone marrow of Bcl2 transgenic mice using antibodies as described[30]. Cells were sorted on expression of c-Kit, Sca-1, low levels of Thy-1.1 and low to negative levels of lineage markers (Lin) using clonecyte software and the single cell deposition unit (Becton Dickinson). See Supplementary Information.

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Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukaemia

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Acute lymphoblastic leukaemia (ALL) is the commonest form of childhood malignancy, and most cases arise from B-cell clones arrested at the pre-B-cell stage of differentiation[1,2]. The molecular events that arrest pre-B-cell differentiation in the leukaemic pre-B cells have not been well characterized. Here we show that the differentiation regulator SLP-65 (an adaptor protein also called BLNK or BASH[3–6]) inhibits pre-B-cell leukaemia in mice. Reconstitution of SLP-65 expression in a SLP-$65^{-/-}$ pre-B-cell line led to enhanced differentiation *in vitro* and prevented the development of pre-B-cell leukaemia in immune-deficient mice. Tyrosine 96 of SLP-65 was required for this activity. The murine SLP-$65^{-/-}$ pre-B-cell leukaemia resembles human childhood pre-B ALL. Indeed, 16 of the 34 childhood pre-B ALL samples that were tested showed a complete loss or drastic reduction of SLP-65 expression. This loss is probably due to the incorporation of alternative exons into SLP-65 transcripts, leading to premature

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